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(57) Abstract

The invention concerns materials and methods relating to an ATP-sensitive potassium ion channel (a K-ATP channel) molecule (and subunits thereof) which is implicated in a number of disease states associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity.

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K-ATP CHANNEL PROTEIN AND METHODS RELATING TO IT.

The present invention concerns materials and methods relating to cell membrane proteins. The cell membrane proteins of particular interest relate to an ATP-sensitive potassium ion channel (a K-ATP channel) molecule (and subunits thereof) which is implicated in a number of disease states associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity.

K-ATP channels are found in many tissues where they play important roles in both physiological and pathophysiological conditions. Basically they couple cell metabolism to electrical activity and play important roles in the physiology and pathophysiology of many tissues. Examples of physiological conditions include insulin secretion, whereas pathophysiological conditions include cardiac and cerebral ischaemia. These tissues include: pancreatic β-cells; smooth, skeletal and cardiac muscle; neurones, including those of the cortex, hippocampus, neocortex, olfactory bulb, cerebellum, substantia nigra (pars reticulata and pars compacta) and respiratory neurones; axons; epithelial cells including kidney tubules. The functional role of the K-ATP channel has been reviewed previously (Ashcroft F.M. (1988) Ann Rev. Neurosci 11, 97-118, Ashcroft F.M. et al., (1990) Cell Sig. 2, 197-214). Its particular role in the pancreatic β-cell has been described (Ashcroft F.M. and Rorsman, P., Prog. Biophys. Mol. Biol. (1990) 54, 87-143) and also it has been found to be effective in substantia nigra neurones.

In pancreatic β-cells, K-ATP channels mediate insulin secretion both in response to glucose (the primary physiological stimulus) and to clinically important drugs. The effects of glucose are mediated indirectly, as a consequence of its metabolism, whereas drugs interact directly with the channel. Metabolic regulation of β-cell K-ATP channels is thought to be mediated by changes in intracellular ATP and MgADP levels which inhibit and activate the channel, respectively. Glucose metabolism elevates intracellular ATP and concomitantly lowers intracellular ADP. This closes K-ATP channels in the β-cell plasma membrane, producing a membrane depolarisation which activates voltage-dependent Ca^{2+} channels, enhances Ca^{2+} influx and triggers insulin release.

The most characteristic property of K-ATP channels is that they are inhibited by an increase in the intracellular ATP concentration. They may also be regulated by hormones and transmitters (as in smooth muscle). K-ATP channels are inhibited by sulphonylurea drugs (such as glibenclamide and tolbutamide) which are used in the treatment of non-insulin-dependent diabetes mellitus (Ashcroft F.M. and Ashcroft S.J.H., Biochem Biophys Acta 1175, 45-49 (1992)) to stimulate insulin release. These drugs bind to a different protein, the sulphonylurea receptor (SUR eg SUR1 or SUR2) which interacts with the pore subunit of the K-ATP channel to bring about its closure, and thereby stimulate insulin secretion. In contrast, other drugs such as diazoxide act as K-ATP channel openers and therefore inhibit insulin release. Diazoxide has been used to treat persistent hyperinsulinaemic hypoglycaemia of infancy, a disease associated with unregulated insulin secretion.

Since the β-cell K-ATP channel plays a central role in glucose-stimulated insulin secretion, it is likely that mutations in this channel might be associated with diabetes mellitus. K-ATP channels also play important roles in the response to cardiac and

cerebral ischaemia and may be implicated in a number of disease states detailed more fully below where there is an abnormal coupling of cellular metabolism and electrophysiological activity.

The regulation of the β-cell K-ATP channel by adenine nucleotides is extremely complex and probably involves several different sites of action. In addition to its well-known inhibitory effect, MgATP enhances K-ATP channel activity as evidenced by the fact that when MgATP is removed, channel activity is greater than that recorded in the control solution prior to application of the nucleotide. This 'refreshment' of channel activity is not observed in the absence of Mg^{2+}, nor is it supported by non-hydrolysable ATP analogues, indicating that MgATP hydrolysis is required. ADP also has both stimulatory and inhibitory actions. In the absence of Mg^{2+}, ADP blocks channel activity. When Mg^{2+} is present, however, high concentrations of ADP are inhibitory whereas low concentrations potentiate channel activity. This suggests that MgADP both activates and inhibits channel activity and that the inhibitory effect dominates in Mg-free solutions or at high MgADP concentrations. There is evidence that nucleotides also modulate the response of the K-ATP channel to drugs. The K-channel opener diazoxide, for example, antagonises the inhibitory effects of MgATP on the β-cell K-ATP channel. In β-cells, the drug has no effect, or is even inhibitory, in the absence of internal Mg^{2+} or when ATP is replaced by non-hydrolysable ATP analogues. This result has been used to support the idea that the action of diazoxide requires protein phosphorylation. However, diazoxide is also effective in the presence of hydrolysable ADP, suggesting that it is more likely that the effect of the drug requires nucleotide hydrolysis rather than phosphorylation.

There has been much debate as to whether SUR and the K-ATP channel are the same or separate proteins, whether SUR confers ATP-sensitivity on an ATP-insensitive pore forming subunit, and whether sulphonylureas can also modulate other types of K-channels.

Cloning of a 140kD high-affinity sulphonylurea receptor (SUR1) from insulinoma cells revealed it to be a member of the ATP-binding cassette (ABC) transporter family, with 2 nucleotide binding folds (Aguilar-Bryan L. et al., 1995 Science, 268, 423-425).

As a result of electrophysiological studies (Ämmälä,C. et al., 1996 J. Physiol. 494,3, 709-714), the inventors have shown that SUR1 does not possess intrinsic channel activity suggesting that SUR alone does not form the K-ATP channel. Instead, it endows sulphonylurea sensitivity on several types of inwardly-rectifying K-channels. It does not, however, necessarily confer ATP-sensitivity on these channels.

The properties of the K-ATP channel suggest that it belongs to the superfamily of inwardly-rectifying potassium channels or Kir channels (Doupnik C.A. et al., Curr. Op. Cell Biol. (1995) 5, 268-278). At least six distinct subfamilies of Kir channels have been cloned. Like these cloned channels, the native K-ATP channel shows inward rectification, little time- or voltage dependence, and is strongly K-selective (Ashcroft and Ashcroft et al., supra). It has been suggested previously that a number of Kir channels that have been cloned constitute the K-ADP channel including Kir1.1a (Ho K. et al., Nature, 362, 31-39 (1993) and Kir3.4 (Ashford M.L.J. et al., Nature, 370, 456-459 (1994)). A further paper reporting the cloning of a K-ATP channel (Kir6.1) is Inagaki et

al., J Biol. Chem. 270, 5691-5694 (1995). However none of these Kir channels have a tissue distribution similar to that of the native K-ATP channel.

The inventors have found that both Kir6.1 and Kir1.1a can couple to SUR1 and they decided to try to use the Kir6.1 and Kir1.1a clones as probes to screen a murine β-cell cDNA library. The approach resulted in the cloning of the mouse gene for an inwardly-rectifying K-channel (Kir6.2) which as shown below comprises the pore forming subunit of the K-ATP channel. The tissue distribution of this gene was determined by Northern blotting. The inventors found that the Kir6.2 gene is expressed in skeletal muscle, heart, brain and insulinoma cells, weakly expressed in lung and very weakly expressed in kidney. No hybridization was detected in spleen, liver and testis. This tissue distribution indicates that Kir6.2 will play important roles in the tissues in which it is expressed. Further the inventors realised that the tissue distribution of Kir6.2 indicates that it may form part of the K-ATP channel as this shows the same tissue distribution as Kir6.2.

The inventors have also cloned the human gene for the Kir6.2 channel and identified its chromosomal location as Chromosome 11p15.1.

K-ATP channels are activated in the brain in response to reduced cerebral metabolism. The inventors show that Kir6.2 and SUR1 MRNAs are abundantly expressed in the brain and have an overlapping localisation at the cellular level using in situ hybridisation histochemistry.

Further using single-stranded conformational polymorphism (SSCP) analysis, the inventors also screened for mutations in the human Kir6.2 gene. A number of variants were identified, one of which is associated with impaired insulin sensitivity and is likely to be associated with the development of insulin resistance.

The electrophysiological properties of the Kir6.2 channel have been characterised and it has been found that it couples to the sulphonylurea receptor SUR1 to form a Kir6.2/SUR1 channel complex. The primary structure of Kir6.2 indicates that it acts as the pore-forming subunit of this channel complex. It also appears that the complex corresponds to the native K-ATP channel .

The results presented hereafter demonstrate that Kir6.2 encodes an inwardly-rectifying ATP-sensitive K-channel subunit which is strongly expressed in pancreatic ß-cells, brain, heart and skeletal muscle. This tissue distribution echoes that of the native K-ATP channel and indicates that Kir6.2 forms part of this channel. Since a similar tissue distribution is found for sulphonylurea binding, it appears that SUR and Kir6.2 couple in native cells.

Although the inventors were unable to detect a significant increase in whole-cell currents in HEK293 cells transfected with Kir6.2 alone, very large currents were observed when Kir6.2 was cotransfected with SUR. This indicates that Kir6.2 does not express functional channels in HEK293 cells and that it couples in some way to SUR to form a functional channel complex similar results were observed when Xenopus oocytes were used as an expression system. It remains unclear whether SUR simply facilitates the insertion of Kir6.2 into the membrane, whether it is needed for channel activity or

whether both processes are involved. Mutations in SUR result in persistent hyperinsulinaemia of infancy (PHHI), a disease associated with unregulated insulin secretion (Thomas et. al. Science (1995) 268, 425 - 429). The findings that SUR is essential for functional Kir6.2 activity suggests that mutations in SUR produce a loss of K-ATP channel activity in PHHI patients, which would be expected to depolarise the ß-cell and thus cause insulin secretion in the absence of secretagogues. Indeed, ß-cells from PHHI patients have been reported to lack K-ATP channel activity (Dunne et. al., Diabetologia (1995) 38, A15). It appears therefore that mutations in either SUR or Kir6.2 may also account for the *decreased* insulin secretion observed in non-insulin-dependent diabetes mellitus.

Kir6.2/SUR currents show no time-dependent activation kinetics and weak inward rectification in the presence of 1.2mM intracellular Mg^{2+}, is consistent with the fact that the second transmembrane region contains an asparagine at position 160, and that position 212 is occupied by serine. The equivalent residues are negatively charged in strong inward rectifiers (Yang et. al., Neuron (1995) 14, 1047-1054).

A marked time-dependent increase in the amplitude of Kir6.2/SUR currents was observed following dialysis with an intracellular solution containing 0.3 mM ATP, but not with 5 mM ATP, suggesting that the current is activated by the washout of ATP from the cell. In addition direct inhibition of single-channel currents by ATP applied at the inner face of the membrane was noted. Since Kir6.2 possesses no obvious consensus sequence for ATP-binding, and SUR has two ATP-binding domains (Aguilar Bryan L. et al., supra) is possible that ATP-sensitivity is conferred by the SUR subunit of the channel complex. Alternatively, a third protein, endogenously expressed in HEK293 cells, may confer ATP-sensitivity.

It has been found that SUR can confer sulphonylurea and diazoxide sensitivity on other types of Kir channels, such as Kir1.1a and Kir6.1, although it is not essential for expression of these channels. This indicates that the sulphonylurea and K channel opener sensitivity of Kir6.2/SUR currents is conferred by the SUR subunit of the complex.

The tissue distribution, single-channel conductance, kinetics, rectification properties, K-selectivity, inhibition by ATP and pharmacological properties of the Kir6.2/SUR complex are all consistent with those of native K-ATP channels. This indicates that the K-ATP channel consists of a complex of Kir6.2 and SUR, with Kir6.2 acting as the pore-forming subunit and SUR acting as a regulator subunit which confers sulphonylurea, and possibly also ATP-sensitivity.

Both Kir6.2 and SUR subunits are required to form a functional K-ATP channel and, unlike many other inward rectifier channels, Kir6.2 does not form functional channels in the absence of the sulphonylurea receptor. SUR1 has two groups of putative transmembrane domains (9+4), each of which is followed by a large cytoplasmic loop which contains a consensus sequence for nucleotide binding. Each nucleotide binding domain (NBD) contains a highly conserved Walker A (W_A) and Walker B (W_B) motif. Studies of many ATP-ases and ABC transporters have shown that these motifs catalyse ATP hydrolysis. An aspartate in the W_B motif co-ordinates the Mg^{2+} ion of MgATP and is required for nucleotide binding, while a lysine in the W_A motif interacts with the Y and

B phosphate groups of ATP and is essential for ATP hydrolysis.

The presence of the NBDs in SUR1 raises the possibility that these may constitute one or more of the sites at which nucleotides regulate K-ATP channel activity. In support of this idea, recent studies have indicated that the second NBD (NBD2) plays an important role in the modulation of K-ATP channel activity by MgADP (Nichols, C.G., et al (1996) Science 272, 1785-1787). Mutation of the W_B aspartate (D1506) in NBD2, for example, removes the ability of MgADP to stimulate channel activity. However, there is evidence that nucleotide hydrolysis is required for the effects of both MgADP and diazoxide on K-ATP channel activity. The inventors examined the effects of mutating the critical lysine in the W_A motifs of either NBD1 (K719A) or NBD2 (K1385M), or both (K719A/K1385M), of SUR1, on K-ATP currents heterologously expressed in *Xenopus* oocytes. They found that hydrolysis of MgATP at NBD1 (but not NBD2) is essential for channel activation by diazoxide. The stimulation of channel activity by MgADP may also involve nucleotide hydrolysis since none of the mutant channels were potentiated by MgADP and, in wild-type channels, neither non-hydrolysable ADP analogues nor ADP in the absence of Mg^{2+} were effective. Mutant currents were slightly more sensitive to ATP than wild-type currents demonstrating that the W_A lysines are not involved in nucleotide inhibition and suggesting that MgATP hydrolysis may partially relieve the inhibitory action of ATP on wild-type currents. Metabolic inhibition led to activation of wild-type and K1385M currents, but not K719A or K719A/K1385M currents, suggesting there may be a factor in addition to ATP and ADP which regulates channel activity. These studies clarify the mechanism of action of nucleotides on K-ATP channels and demonstrate that the W_A lysines of SUR1 play an essential role in the channel activation by MgADP and diazoxide.

In the light of the above, the following regions of the Kir6.2 are of particular interest for the reasons summarized below. In particular, they are targets for the investigation of mutations giving rise to, or associated with diseases, or alternatively are important for ATP-sensitive K-channel activity and therefore may be targeted by drugs in order to modify that activity. The fragments of the Kir6.2 molecule comprising these regions together with nucleotide sequences which encode them form preferred embodiments of the invention:

1. *The regions which make up the pore-forming domains.*
The selectivity filter and the inner part of the pore is formed by the H5 region of Kir6.2 indicated on Fig. 2. Mutations in this region may be expected to produce alterations in the channel selectivity and conductance, and thus confer disease.

2. *The intracellular mouth and intracellular vestibule of the pore.*
This may be involved in the actions of drugs which activate or inhibit channel activity. The Kir6.2 channel and the sulphonylurea receptor make up the K-ATP channel complex, with Kir6.2 comprising the pore-forming subunit and SUR forming the drug-binding subunit. It is possible that sulphonylurea (and ATP) inhibition of channel activity is conferred by the movement of part of the sulphonylurea receptor into the intracellular mouth of the channel, which thereby blocks movement of ions. Alternatively, channel activity may be blocked by part of the Kir6.2 molecule which interacts with the pore and SUR may prevent this interaction. This would explain why both subunits are required for

functional K-ATP channel activity. In this case ATP and sulphonylurea would block channel activity by interfering with the interaction between Kir6.2 and SUR and so cause channel closure. In both cases, the conformational change in SUR which causes this effect is brought about by the binding of ATP or sulphonylureas: ATP probably binds to one or both of the two nucleotide binding domains of SUR; sulphonylureas may also bind at one or other of these sites or they may bind to other sites which allostearically influence the nucleotide binding domain or to other sites in the protein. It appears that K-channel openers displace ATP from its binding site of SUR, or interfere with the binding of ATP, or enhance the stimulatory action of MgATP or MgADP and thereby reverse or prevent the conformational change in SUR which causes channel inhibition. The intracellular mouth of the pore is likely to be formed from Kir6.2, but there may be additional contributions from SUR. The intracellular mouth of the channel is therefore the target site with which the inhibitory domain of SUR interacts to block ion flux.

Clearly, drugs which interact with Kir6.2 in the same way as the inhibitory or stimulatory domain of SUR they could be used as novel inhibitors of K-ATP channel activity. In this respect the intracellular mouth of the channel is a prime target. Thus this region of the protein will be useful in screening for such inhibitors for example for use in therapy.

3. *The extracellular loop of the channel.*
This may be important for the binding of antibodies, which could be involved in diseased states. Hence this region may be used to simulate or generate therapeutic antibodies or antibodies for immunocytochemistry or screening methods.

4. *The transmembrane domains.*
The results obtained by the applicants suggest that Kir6.2 does not show functional activity in the absence of SUR. By analogy with what is known of other Kir channels, it appears that the transmembrane domains are critical for whether a channel is targeted to the membrane or is degraded. Thus SUR may interact with the transmembrane domains of Kir6.2 to ensure that Kir6.2 reaches the membrane correctly. If this interaction with SUR is disturbed in some way then it could result in incorrect processing of Kir6.2 and thus disease states in which Kir6.2 is underexpressed (such as PHHI, in the β-cell). Correction of this processing defect could lead to correct expression of Kir6.2 and thus ameliorate the disease.

The inventors also provide herein the human Kir6.2 promoter (see example 11 and Fig.12). Mutations or abnormalities in the promoter of Kir6.2 may lead to under or over-expression of the protein and thus to differences in the level of K-ATP channel activity. This may be reasonably expected to result in human (or animal disease) as listed below.

Knowledge of this promoter sequence is therefore of use in the diagnosis of these diseases. Further agents designed to interfere with the promoter sequence and affect Kir6.2 expression may be of therapeutic benefit. The promoter sequence may also be of use in the generation of genetically engineered animals (eg mice) with either enhance of expression or underexpression of Kir6.2 (ie in transgenic and knock out animals).

Thus diagnostic applications and screening for drugs as generally described hereafter in

relation to the Kir6.2 sequences as shown in Figs. 1 and 7 may also be carried out by use of part or all of the human Kir6.2 promoter the sequence of which is provided in Fig. 12.

According to one aspect of the present invention there is provided a nucleic acid molecule which has a nucleotide sequence encoding an ATP-sensitive K-channel protein or a subunit thereof. The protein may comprise a pore subunit of an ATP-sensitive K-channel. The protein may be able to complex with a SUR (eg SUR1). The protein complexed with SUR may have ATP-sensitive K-channel activity. The protein may include an amino acid sequence as shown in Fig.1 or Fig.7.

The nucleotide coding sequence may be that shown in Fig.1 or Fig. 7, or it may be a mutant, variant, derivative, allele or fragment of the sequences shown. The sequence may differ from those shown by a change which is one or more of addition, insertion, deletion and substitution of one or more nucleotides of the sequences shown. Changes to a nucleotide sequence as shown may result in an amino acid change at the protein level, or not, as determined by the genetic code.

Thus, nucleic acid according to the present invention may include a nucleotide sequence different from those sequences shown in Figs 1 and 7 which yet still encodes an ATP-sensitive K-channel protein such as a pore subunit of an ATP-sensitive K-channel or a protein which is able to complex with a SUR (eg SUR1) and when so complexed has ATP-sensitive channel activity.

Thus, nucleic acid according to the present invention may include a nucleotide sequence different from the sequences shown in Fig. 1 or Fig.7 yet which encodes a protein with the same amino acid sequence as shown in a said Fig.

On the other hand, the encoded protein may comprise an amino acid sequence which differs by one or more amino acid residues from the amino acid sequences shown in Fig.1 or Fig.7.

Nucleic acid encoding an ATP-sensitive K-channel protein such as a pore subunit of an ATP-sensitive K-channel (eg Kir6.2) or a protein which is able to complex with a SUR (eg SUR1)and when so complexed has ATP-sensitive channel activity, such a protein being an amino acid sequence mutant, variant, derivative or allele of an amino acid sequence as shown in Fig.1 or Fig.7 is further provided by the present invention. Such proteins are discussed below.

In relation to the above it is to be stated that Kir6.2 may couple to different kinds of SUR. Thus Kir6.2 may be complexed with SUR1, SUR2 and their splice variants to other SUR family members and to other members of the ABC transporter family eg the cystic fibrosis transmembrane conductance regulator (CFTCR) or multi-drug resistance related protein (MDRRP). Thus references to SUR herein may be construed as being a reference to any of the SUR family members or members of the ABC transporter family. Thus references to Kir6.2/SUR or references to SUR should be construed as covering all forms of SUR including SUR1, SUR2 and splice variants.

Nucleic acid encoding such a protein may show 50% or greater homology with a

nucleotide coding sequence as shown in Fig.1 or Fig.7, and/or greater than about 60% homology, and/or greater than about 70% homology, and/or greater than about 80% homology, and/or greater than about 95% homology.

Particular alleles according to the present invention have sequences encoding polymorphisms as set out in eg Example 6 hereafter. The allele coding for valine at amino acid residue 270 instead of leucine has been shown by the experimental work described herein to be associated with higher fasting insulin concentration. Implications for screening, e.g. for diagnostic or prognostic purposes, are discussed below.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA.

The coding sequence shown in Figs 1 and 7 is a DNA sequence. For the RNA equivalent, T is substituted with U.

Nucleic acid may be provided as part of a replicable vector. Therefore also provided by the present invention are vectors including nucleic acid as set out above, particularly any expression vector from which the encoded protein can be expressed under appropriate conditions. Also provided are host cells containing any such vectors or nucleic acids. Host cells may be Xenopus oocytes. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a protein of interest and appropriate regulatory sequences for expression of the protein, in an *in vitro* expression system, e.g. reticulocyte lysate, or *in vivo*, e.g. in eukaryotic cells such as COS or CHO cells or in prokaryotic cells such as *E. coli*. This is discussed further below.

The nucleic acid sequences provided in Figs 1 and 7 are useful for identifying a nucleic acid of interest (and which may be according to the present invention) in a test sample. The present invention provides a method of obtaining nucleic acid of interest, the method including hybridisation of a probe having a sequence as shown in Fig.1 or Fig 7 or a complementary sequence, to target nucleic acid.

Hybridisation is generally followed by identification of successful hybridisation and isolation of nucleic acid which has hybridised to the probe. This may involve one or more steps of PCR.

It will not usually be necessary to use a probe with the complete sequence shown in Fig 1 or Fig 7. Shorter fragments, particularly fragments with a sequence conserved between the mouse and human sequences (Figs 1 and 7 respectively) may be used.

Nucleic acid according to the present invention is obtainable using one or more oligonucleotide probes or primers designed to hybridise with one or more fragments of a nucleic acid sequence as shown in Fig 1 or Fig 7, particularly fragments of relatively rare sequence, based on codon usage or statistical analysis. A primer designed to hybridise

with a fragment of a nucleic acid sequence as shown in Fig.1 or Fig 7 may be used in conjunction with one or more oligonucleotides designed to hybridise to a sequence in a cloning vector within which target nucleic acid has been cloned, or in so-called "RACE" (rapid amplification of cDNA ends) in which cDNA's in a library are ligated to an oligonucleotide linker and PCR is performed using a primer which hybridises with a sequence shown in Fig.1 or Fig 7 and a primer which hybridises to the oligonucleotide linker.

Nucleic acid isolated and/or purified from one or more cells (e.g. human) or a nucleic acid library derived from nucleic acid isolated and/or purified from cells (e.g. a cDNA library derived from mRNA isolated from the cells), may be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR) (reviewed for instance in "PCR protocols; A Guide to Methods and Applications", Eds. Innis *et al*, 1990, Academic Press, New York). PCR comprises steps of denaturation of template nucleic acid (if double-stranded), annealing of primer to target, and polymerisation. The nucleic acid probed or used as template in the amplification reaction may be genomic DNA, cDNA or RNA.

In the context of cloning, it may be necessary for one or more gene fragments to be ligated to generate a full-length coding sequence. Also, where a full-length encoding nucleic acid molecule has not been obtained, a smaller molecule representing part of the full molecule, may be used to obtain full-length clones. Inserts may be prepared from partial cDNA clones and used to screen cDNA libraries. The full-length clones isolated may be subcloned into expression vectors and activity assayed by transfection into suitable host cells, e.g. with a reporter plasmid.

A method may include hybridisation of one or more (e.g. two) probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as part of a PCR procedure, or as part of a probing procedure not involving PCR. An example procedure would be a combination of PCR and low stringency hybridisation. A screening procedure, chosen from the many available to those skilled in the art, is used to identify successful hybridisation events and isolated hybridised nucleic acid.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include examination of restriction fragment length polymorphisms (RFLPs), amplification using PCR, RNAase cleavage and allele specific oligonucleotide probing.

Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells. Preliminary experiments may be performed

by hybridising under low stringency conditions various probes to Southern blots of DNA digested with restriction enzymes. Suitable conditions would be achieved when a large number of hybridising fragments were obtained while the background hybridisation was low. Using these conditions nucleic acid libraries, e.g. cDNA libraries representative of expressed sequences, may be searched.

Those skilled in the art are well able to employ suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on.

On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived. An oligonucleotide for use in nucleic acid amplification may have about 10 or fewer codons (e.g. 6, 7 or 8), i.e. be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length, but not more than 18-20. Those skilled in the art are well versed in the design of primers for use processes such as PCR.

Preferred amino acid sequences suitable for use in the design of probes or PCR primers may include sequences conserved (completely, substantially or partly) between the mouse and human sequences shown respectively in Figs 1 and 7.

A further aspect of the present invention provides an oligonucleotide or polynucleotide fragment of the nucleotide sequence shown in Fig 1 or Fig 7, or a complementary sequence, in particular for use in a method of obtaining and/or screening nucleic acid. Some preferred oligonucleotides have a sequence as given in eg Examples 6 and 7 below or a sequence which differs from any of the sequences given by addition, substitution, insertion or deletion of one or more nucleotides, but preferably without abolition of ability to hybridise selectively with nucleic acid with the sequence shown in Fig 1 or Fig 7, that is wherein the degree of homology of the oligonucleotide or polynucleotide with one of the sequences given is sufficiently high.

In some preferred embodiments, oligonucleotides according to the present invention that are fragments of any of the sequences shown in Fig 1 or Fig 7 or a mutant, variant, derivative or allele thereof, are at least about 10 nucleotides in length, more preferably at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length. Such fragments themselves individually represent aspects of the present invention.

Oligonucleotide probes or primers, as well as the full-length sequence (and mutants, alleles, variants and derivatives) are also useful in screening a test sample containing nucleic acid for the presence of alleles, mutants and variants, with diagnostic and/or prognostic implications as discussed in more detail below.

Thus, the invention provides a gene probe or primer useful in diagnosing a disease associated with abnormal coupling of cellular metabolism and electro-physiological activity because of a mutation in a gene associated with the ATP sensitive K-channel gene. The mutation may be in the Kir6.2 gene or in the gene for a SUR (eg SUR1). If

in a SUR, the mutation may be in a coding sequence for lysine in the motif W_A or a coding sequence for lysine in the W_B motif. Where one is examining for a mutation in the Kir6.2 gene the probe or primer may comprise a fragment of either a sequence as shown in Fig 1 or Fig 7 or a sequence complementary to a sequence as shown in Fig 1 or Fig 7. Examples of such fragments include short sequences derived from or around the region of naturally occurring mutations which are characteristic of certain disease states, for example those disease states listed below. Such fragments, which suitably range in size from 15-200 bases can be used as gene probes in the diagnosis of a disease state. Particular probes for use in the diagnosis of particular diseases will be determinable using conventional methods. The fragment may include a region of a mutation so that diagnosis may be effected by detecting mismatches between the fragment and the said region. Alternatively, the fragment may comprise a flanking sequence with respect to the mutation, such that the fragment may be used as a primer for example for use in techniques such as the polymerase chain reaction (PCR). In particular, the invention provides a primer designed for amplification of human genomic DNA for use in the screening of mutations in the Kir6.2 gene which comprises a fragment of a nucleotide sequence substantially as shown in Fig 1 or Fig 7.

Nucleic acid according to the present invention may be used in methods of gene therapy, for instance in treatment of individuals with the aim of preventing or curing (wholly or partially) diseases associated with abnormal coupling of cellular metabolism and electrical activity. This too is discussed below.

Nucleic acid according to the present invention, such as a full-length coding sequence or oligonucleotide probe or primer, may be provided as part of a kit, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may include instructions for use of the nucleic acid, e.g. in PCR and/or a method for determining the presence of nucleic acid of interest in a test sample. A kit wherein the nucleic acid is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc. The nucleic acid may be labelled. A kit for use in determining the presence or absence of nucleic acid of interest may include one or more articles and/or reagents for performance of the method, such as means for providing the test sample itself, e.g. a swab for removing cells from the buccal cavity or a syringe for removing a blood sample (such components generally being sterile).

In relation to the Kir6.2 promoter provided herein, the present invention provides further materials and methods. Thus there is provided a nucleic acid molecule which has a nucleotide sequence which is able to function as a promoter for the expression of an ATP-sensitive K-channel protein or a subunit of such a K-channel protein. The nucleotide sequence may be that shown in Fig. 12 or it may be a mutant, variant, derivative, allele or fragment of the sequence shown in Fig. 12 which retains functional activity as a promoter. The sequence may differ in a way as stated previously in relation to mutants etc of the sequences shown in Fig. 1 or Fig. 7 and the other general comments in relation to the features and uses of the sequences of Figs. 1 and 7 should also be interpreted as being applicable to a nucleic acid molecule as represented by Fig. 12.

In particular a promoter nucleic acid molecule as provided may be part of a replicable

vector. The sequence of Fig. 12 can also be used to identify other related sequences of interest in a test sample by using it as the basis of probes/primers.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene facilitates determination of promoter activity by reference to protein production.

Further provided by the present invention is a nucleic acid construct comprising a **Kir6.2** promoter region or a fragment, mutant, allele, derivative or variant thereof able to promote transcription, operably linked to a heterologous gene, e.g. a coding sequence. Generally, the gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following expression. A gene whose encoded product may be assayed following expression is termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β-galactosidase and luciferase. β-galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectrophotometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine gene activity. Any suitable reporter/assay may be used and it should be appreciated that no particular choice is essential to or a limitation of the present invention.

Nucleic acid constructs comprising a promoter (as disclosed herein) and a heterologous gene (reporter) may be employed in screening for a substance able to modulate activity of the promoter. For therapeutic purposes, (e.g. for treatment of a disease condition as noted herein) a substance able to up- or down-regulate expression of the promoter may be sought. A method of screening for ability of a substance to modulate activity of a promoter may comprise contacting an expression system, such as a host cell, containing a nucleic acid construct as herein disclosed with a test or candidate substance and determining expression of the heterologous gene.

The level of expression in the presence of the test substance may be compared with the level of expression in the absence of the test substance. A difference in expression in the presence of the test substance indicates ability of the substance to modulate gene expression. An increase in expression of the heterologous gene compared with expression of another gene not linked to a promoter as disclosed herein indicates specificity of the substance for modulation of the promoter.

A promoter construct may be introduced into a cell line using any technique previously described to produce a stable cell line containing the reporter construct integrated into the genome. The cells may be grown and incubated with test compounds for varying times. The cells may be grown in 96 well plates to facilitate the analysis of large numbers of compounds. The cells may then be washed and the reporter gene expression analysed. For some reporters, such as luciferase the cells will be lysed then analysed.

Following identification of a substance which modulates or affects promoter activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified using a nucleic acid molecule as a modulator of promoter activity, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for increasing or decreasing expression of Kir6.2 expression for instance in treatment (which may include preventative treatment) of a disease condition as noted, use of such a substance in manufacture of a composition for administration to patients, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A further aspect of the present invention provides a polypeptide which comprises part or all of an ATP-sensitive K-channel. Thus a polypeptide may comprise a subunit of an ATP-sensitive K-channel or a polypeptide which is able to complex with SUR (eg SUR1) and when so complexed has ATP-sensitive K-channel activity. The subunit may have a pore. The polypeptide may have an amino acid sequence as shown in Fig 1 or Fig 7. A polypeptide may be in isolated and/or purified form, free or substantially free of material with which it is naturally associated, such as other polypeptides or such as human polypeptides or (for example if produced by expression in a prokaryotic cell) lacking in native glycosylation, e.g. unglycosylated.

Polypeptides which are amino acid sequence variants, alleles, derivatives, mutants or fragments in relation to a polypeptide as stated above are also provided by the present invention. A polypeptide which is a variant, allele, derivative, mutant or fragment may have an amino acid sequence which differs from that given in Fig 1 or Fig 7 by one or more of addition, substitution, deletion and insertion of one or more amino acids. Preferred such polypeptides have the electrophysiological and/or immunological characteristics of an ATP-sensitive K-channel. Thus they may comprise a pore member subunit of an ATP-sensitive K-channel. They may be able to complex with a SUR (eg

SUR1). The polypeptide when complexed with a SUR may have ATP-sensitive K-channel activity. Such variant etc polypeptides may in addition to the features described above, or alternatively, have immunological cross-reactivity with an antibody reactive the polypeptide for which the sequence is given in Fig 1 or Fig 7.

A polypeptide which is an amino acid sequence variant, allele, derivative, mutant or fragment of the amino acid sequence shown in Fig 1 or Fig 7 may comprise an amino acid sequence which shares greater than about 35% sequence identity with the sequence shown, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 60% similarity, greater than about 70% similarity, greater than about 80% similarity or greater than about 90% or greater than about 95% similarity with an amino acid sequence as shown in Fig 1 or Fig 7. Particular amino acid sequence variants may differ from that shown in Fig 1 or Fig 7 by insertion, addition, substitution or deletion of 1 amino acid, 2, 3, 4, 5-10, 10-20 20-30, 30-50, 50-100, 100-150, or more than 150 amino acids.

Sequences of amino acid sequence variants representative of preferred embodiments of the present invention are given in the examples. Screening for the presence of one or more of these in a test sample has a diagnostic and/or prognostic use, for instance in diagnosing a disease associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity because of a mutation in a gene associated with the ATP sensitive K-channel such as the gene for Kir6.2 and/or the gene for a SUR (eg SUR1) as mentioned earlier.

A polypeptide according to the present invention may be isolated and/or purified (e.g. using an antibody), for instance after production by expression from encoding nucleic acid (for which see below). Polypeptides according to the present invention may also be generated wholly or partly by chemical synthesis. The isolated and/or purified polypeptide may be used in formulation of a composition, which may include at least one additional component, for example a pharmaceutical composition including a pharmaceutically acceptable excipient, vehicle or carrier. A composition including a polypeptide according to the invention may be used in prophylactic and/or therapeutic treatment as discussed below.

A polypeptide, peptide fragment, allele, mutant or variant according to the present invention may be used as an immunogen or otherwise in obtaining specific antibodies. Antibodies are useful in purification and other manipulation of polypeptides and peptides, diagnostic screening and therapeutic contexts. This is discussed further below.

Identification of the identity of the Kir6.2 gene and therefore also the Kir6.2 channel molecule itself, offers the possibility of a rational approach to drug design. With knowledge of the molecular structure of the channel it may now be possible to design drugs (i) which selectively target different tissues and (ii) which have greater potency. Since the Kir6.2 channel forms a complex with a SUR (eg SUR1), drugs may be targeted not just to the Kir6.2 protein itself, but also to the regions of interaction of between Kir6.2 and the SUR, such as the pore, the pore mouth and the transmembrane domains.

A polypeptide according to the present invention may be used in screening for molecules which affect or modulate its activity or function. In particular one may use either or both of the Kir6.2 and a SUR (eg SUR1) polypeptides in such a screen. Kir6.2 may be used as complexed to a SUR (eg SUR1). Such molecules may be useful in a therapeutic (possibly including prophylactic) context.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in treating or preventing a disease associated with abnormal coupling of cellular metabolism and electro-physiological activity (because of a mutation in a gene associated with the ATP sensitive K-channel, such as the gene for Kir6.2 or the gene for a SUR such as SUR1) is provided by polypeptides according to the present invention. Substances identified as modulators of the polypeptide represent an advance in the fight against such diseases since they provide basis for design and investigation of therapeutics for *in vivo* use.

A method of screening for a substance which modulates activity of a polypeptide may include contacting one or more test substances with the polypeptide/polypeptides/polypeptide complex in a suitable reaction medium, testing the activity of the polypeptide in the presence of the test substance or substances and comparing that activity with the activity of the polypeptide in comparable reaction medium without the test substance or substances. A difference in activity between the treated and untreated polypeptides is indicative of a modulating effect of the relevant test substance or substances. In this context one may screen for modulating substances by examining changes in the properties of Kir6.2 or other ATP-sensitive K channels. Examples of the electrophysiological and pharmacological properties of the cloned K-ATP channel complex (Kir6.2/SUR1 complex) are set forth below.

Thus identification of the Kir 6.2 channel enables the design of simple and specific screening systems for testing the efficacy of drugs on channel activity. Screens which are based upon the use of the entire Kir 6.2 channel protein or nucleotide sequence, or just a fragment of these can be envisaged. For instance, a useful screen could be derived from a fragment of the Kir6.2 channel molecule or functional equivalent, which includes the binding site of the Kir6.2 channel to a SUR and which gives rise to an active Kir6.2/SUR channel complex. Alternatively, a fragment representing the pore structure (shown as H5 in Figure 2) or the pore mouth may be used to assess the effect of a drug in blocking the pore and thereby closing the channel. Such protein fragments, as well as nucleotide sequences which encode them fall within the ambit of the invention.

For example, the gene encoding Kir6.2 or a fragment thereof, for instance a fragment comprising the site of interaction with SUR or a fragment encoding the pore or pore entrances may be transfected (permanently or transiently) into a cell line such as a mammalian cell line, and electrophysiological or binding studies implemented to establish the potency of drug efficacy or drug binding. Cotransfection with regulatory proteins (e.g.

SUR) or channel subunits would enable the interaction between these proteins to be investigated (by flux studies, electrophysiological studies, etc.).

Alternatively, binding studies may be carried out *in vitro*, for example by detecting binding between the Kir6.2 protein or a SUR (eg SUR1) or a polypeptide comprising a region of interaction between Kir6.2 and a SUR (eg SUR1) or a functional equivalent thereof and a drug under test. The studies may be carried out using conventional techniques. For instance, one component of the prospective binding pair may be immobilised on a support means and the other administered to said support. Binding may be detected using conventional techniques using for example a labelling means such as a labelled antibody.

As used herein the expression "functional equivalent" means a peptide which although different in amino acid structure, has a similar biological effect in the context in which the peptide is used.

Novel drugs identified by the screening methods of the invention form yet a further aspect of the invention.

The invention further provides a method of modifying the activity of an ATP sensitive K-channel by (a) interfering with the interaction between Kir6.2 and a SUR (eg SUR1) by administering an agent which blocks said interaction or (b) administering an agent which blocks the channel pore formed by the Kir6.2/SUR complex.

Such agents may be identified by screening for example as described above. They may include blocking fragments of the Kir6.2 or SUR molecules themselves, or functionally equivalent peptides or mimetics and these form a further aspect of the invention.

Combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide.

Prior to, or as well, as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g. in a yeast two-hybrid system (which requires that both the polypeptide and the test substance can be expressed in yeast from the encoding nucleic acid). This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide.

Following identification of a substance which modulates or affects polypeptide activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified using a nucleic acid molecule as a modulator of polypeptide activity, in accordance with what is disclosed herein, but also to a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, use of such a substance in manufacture of a composition for administration, and a method of making a pharmaceutical composition comprising

admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients. Diseases which may benefit from use of the various approaches described herein are listed below.

A substance identified as a modulator of polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics of substances identified as having ability to modulate polypeptide activity using a screening method as disclosed herein are included within the scope of the present invention.

A polypeptide, peptide or substance able to modulate activity of a polypeptide according to the present invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

A convenient way of producing a polypeptide according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system.

Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptides include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*. Also of interest are Xenopus oocytes.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences (see later), terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Ausubel *et al.* eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid may take place *in vivo* by way of gene therapy, as discussed below.

A host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place *in vivo* or *ex vivo*), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention.

This may have a therapeutic aim. The presence of a mutant, allele or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying the role of the gene or substances which modulate activity of the encoded polypeptide *in vitro* or are otherwise indicated to be of therapeutic potential.

Instead of, or as well, as being used for the production of a polypeptide encoded by a transgene, host cells may be used as a nucleic acid factory to replicate the nucleic acid of interest in order to generate large amounts of it. Multiple copies of nucleic acid of interest may be made within a cell when coupled to an amplifiable gene such as DHFR. Host cells transformed with nucleic acid of interest, or which are descended from host cells into which nucleic acid was introduced, may be cultured under suitable conditions, e.g. in a fermentor, taken from the culture and subjected to processing to purify the nucleic acid. Following purification, the nucleic acid or one or more fragments thereof may be used as desired, for instance in a diagnostic or prognostic assay as discussed elsewhere herein.

The provision of the new polypeptides as described herein enables for the first time the production of antibodies able to bind to such polypeptides or polypeptide complexes (eg Kir6.2/SUR1) specifically. Accordingly, a further aspect of the present invention

provides an antibody able to bind specifically to a polypeptide whose sequence is given in Fig 1 or Fig 7. Such an antibody may be specific in the sense of being able to distinguish between the polypeptide it is able to bind and other human polypeptides for which it has no or substantially no binding affinity (e.g. a binding affinity of about 1000x worse). Specific antibodies bind an epitope on the molecule which is either not present, or is not accessible, on other molecules. Antibodies according to the present invention may be specific for the wild-type polypeptide. Antibodies according to the invention may be specific for a particular mutant, variant, allele, derivative or fragment polypeptide as between that molecule and the wild-type polypeptide, so as to be useful in diagnostic and prognostic methods as discussed below. Antibodies are also useful in purifying the polypeptide or polypeptides to which they bind, e.g. following production by recombinant expression from encoding nucleic acid.

Preferred antibodies according to the invention are isolated, in the sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage *et al.*, 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two

Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies according to the present invention may be used in screening for the presence of a polypeptide, for example in a test sample containing cells or cell lysate as discussed, and may be used in purifying and/or isolating a polypeptide according to the present invention, for instance following production of the polypeptide by expression from encoding nucleic acid therefor. Antibodies may modulate the activity of the polypeptide to which they bind and so, if that polypeptide has a deleterious effect in an individual, may be useful in a therapeutic context (which may include prophylaxis).

An antibody may be provided in a kit, which may include instructions for use of the antibody, e.g. in determining the presence of a particular substance in a test sample. One or more other reagents may be included, such as labelling molecules, buffer solutions, elutants and so on. Reagents may be provided within containers which protect them from the external environment, such as a sealed vial.

The identification of the Kir6.2 gene and variations therein and the identification of variations in the nucleotide binding domains of a SUR such as SUR1 paves the way for aspects of the present invention to provide the use of materials and methods for establishing the presence or absence in a test sample of an variant form of the gene, in particular an allele or variant specifically associated with diseases associated with abnormal coupling of cellular metabolism and electrical activity and/or K^+ fluxes. This may be for diagnosing a predisposition of an individual to such a disease. It may be for diagnosing the condition of a patient with the disease as being associated with the K-ATT channel and the Kir6.2 gene and or SUR genes..

This allows for planning of appropriate therapeutic treatment, permitting stream-lining of treatment by targeting those most likely to benefit.

A variant form of the Kir6.2 or a SUR (eg SUR1) gene may contain one or more insertions, deletions, substitutions and/or additions of one or more nucleotides compared with the wild-type sequence which may or may not disrupt the gene function. Differences at the nucleic acid level are not necessarily reflected by a difference in the amino acid sequence of the encoded polypeptide. However, a mutation or other difference in a gene may result in a frame-shift or stop codon, which could seriously affect the nature of the polypeptide produced (if any), or a point mutation or gross mutational change to the encoded polypeptide, including insertion, deletion, substitution and/or addition of one or more amino acids or regions in the polypeptide. A mutation in a promoter sequence or other regulatory region may prevent or reduce expression from the gene or affect the processing or stability of the mRNA transcript.

There are various methods for determining the presence or absence in a test sample of a particular nucleic acid sequence, such as a sequence shown in Fig 1 or Fig 7 or a mutant, variant, fragment or allele thereof.

Tests may be carried out on preparations containing genomic DNA, cDNA and/or mRNA. Testing cDNA or mRNA has the advantage of the complexity of the nucleic acid being reduced by the absence of intron sequences, but the possible disadvantage of extra time and effort being required in making the preparations. RNA is more difficult to manipulate than DNA because of the wide-spread occurrence of RNAases.

Nucleic acid in a test sample may be sequenced and the sequence compared with other known sequences eg wild-type sequences. For example Kir6.2 sequences may be compared with those shown in Fig 1 or Fig 7 to determine whether or not a difference is present. If so, the difference can be investigated for association with the disease state.

Since it will not generally be time- or labour-efficient to sequence all nucleic acid in a test sample, or even the whole Kir6.2 gene, a specific amplification reaction such as PCR using one or more pairs of primers may be employed to amplify the region of interest in the nucleic acid. The amplified nucleic acid may then be sequenced as above, and/or tested in any other way to determine the presence or absence of a particular feature. Nucleic acid for testing may be prepared from nucleic acid removed from cells or in a library using a variety of other techniques such as restriction enzyme digest and electrophoresis.

Nucleic acid may be screened using a variant- or allele-specific probe. Such a probe corresponds in sequence to a region of the gene of interest eg the Kir6.2 gene, or the SUR1 gene or its complement, containing a sequence alteration known to be associated with the disease state. Under suitably stringent conditions, specific hybridisation of such a probe to test nucleic acid is indicative of the presence of the sequence alteration in the test nucleic acid. For efficient screening purposes, more than one probe may be used on the same test sample.

Allele- or variant-specific oligonucleotides may similarly be used in PCR to specifically amplify particular sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may for instance be treated in a way that enables one to display the polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being selected.

An alternative or supplement to looking for the presence of variant sequences in a test sample is to look for the presence of the normal sequence, e.g. using a suitably specific oligonucleotide probe or primer.

Use of oligonucleotide probes and primers has been discussed in more detail above.

Approaches which rely on hybridisation between a probe and test nucleic acid and subsequent detection of a mismatch may be employed. Under appropriate conditions (temperature, pH etc.), an oligonucleotide probe will hybridise with a sequence which is not entirely complementary. The degree of base-pairing between the two molecules will be sufficient for them to anneal despite a mis-match. Various approaches are well known in the art for detecting the presence of a mis-match between two annealing nucleic acid molecules.

For instance, RNAase A cleaves at the site of a mis-match. Cleavage can be detected by electrophoresing test nucleic acid to which the relevant probe or probe has annealed and looking for smaller molecules (i.e. molecules with higher electrophoretic mobility) than the full length probe/test hybrid.

Thus, an oligonucleotide probe that has the sequence of a region of eg the normal Kir6.2 gene (either sense or anti-sense strand) in which mutations associated with a disease state occur may be annealed to test nucleic acid and the presence or absence of a mis-match determined. Detection of the presence of a mis-match may indicate the presence in the test nucleic acid of a mutation associated with susceptibility to the disease state. On the other hand, an oligonucleotide probe that has the sequence of a region of eg the Kir6.2 gene including a mutation associated with susceptibility to a disease state may be annealed to test nucleic acid and the presence or absence of a mis-match determined. The absence of a mis-match may indicate that the nucleic acid in the test sample has the normal sequence. In either case, a battery of probes to different regions of the gene may be employed.

The presence of differences in sequence of nucleic acid molecules may be detected by means of restriction enzyme digestion. One compares the restriction pattern produced by use of a given one or more restriction enzymes on sample containing normal and variant genes.

A test sample of nucleic acid may be provided for example by extracting nucleic acid from cells, e.g. in saliva or preferably blood, or for pre-natal testing from the amnion, placenta or foetus itself.

There are various methods for determining the presence or absence in a test sample of a particular polypeptide, such as the polypeptide with an amino acid sequence as shown in Fig 1 or Fig 7 or an amino acid sequence mutant, variant or allele thereof.

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for one or more particular variants of the polypeptide.

In such cases, the sample may be tested by being contacted with a specific binding member such as an antibody under appropriate conditions for specific binding, before binding is determined, for instance using a reporter system as discussed. Where a panel of antibodies is used, different reporting labels may be employed for each antibody so that binding of each can be determined.

A specific binding member such as an antibody may be used to isolate and/or purify its binding partner polypeptide from a test sample, to allow for sequence and/or biochemical analysis of the polypeptide to determine whether it has the sequence and/or properties of a polypeptide whose sequence is shown in Fig 1 or Fig 7, or if it is a mutant or variant form. Amino acid sequence analysis (using automated sequencing machines) and eg hydrophobicity profiling are routine in the art.

A test sample containing one or more polypeptides may be provided for example as a crude or partially purified cell or cell lysate preparation, e.g. using cells from saliva or preferably blood, or for pre-natal testing from the amnion, placenta or foetus itself.

In relation to therapy, whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the

present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

The administration may be systemic or targeted, the latter employing direct (eg topical) application of the therapeutic agent to the target cells or the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they could be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells.

Alternatively, the agent could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated.

This type of approach is sometimes known as ADEPT or VDEPT; the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, eg an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

Mutations in K-channel may lead to human diseases such as diabetes mellitus including non-insulin-dependent diabetes; maturity-onset-diabetes of the young and insulin-dependent diabetes; diseases which result in increased insulin secretion such as persistent hyperinsulinaemia of infancy; hypertension and hypotension; cerebral ischaemia; stroke; cerebral vasospasm; peripheral arterial disease; vascular smooth muscle disease; angina pectoris; long QT syndrome; vasospastic angina; cardiac dysrhythmia; ischaemia induced cardiac arrhythmias; Parkinson's Disease; diseases associated with abnormal cerebellar function (eg ataxia) asthma; diseases of intestinal smooth muscle, including irritable bowel syndrome; urinary incontinence; male alopecia; certain skeletal muscle myopathies, such as those associated with membrane depolarisation, hyperpolarisation or hypopolarisation; appetite (both reduced and excessive); abnormalities and diseases of uterine smooth muscle, including premature labour; diseases involving abnormal nerve conduction; inappropriate secretion of renin and insensitivity to certain classes of drugs (eg sulphonylureas, K-channel openers etc) caused by inappropriate coupling between Kir6.2 and the drug-binding subunits of the channel complex.

Identification of the Kir6.2 channel gene and its chromosomal location is therefore of use in the diagnosis of the above-mentioned diseases. Likewise is the identification of variations in the W_A and W_B motifs in the nucleotide binding domains of a SUR (eg SUR1). A mutation giving rise to a particular disease may be identified using known investigative techniques such as linkage analysis of families, and characterisation of mutations using SSCP and/or direct sequencing. This will then enable patients to be genetically screened for such diseases. Methods of diagnosing diseases caused or associated with mutations in the K-ATP channel or incorrect regulation of its activity, in particular diabetes, using the gene sequence information form a further aspect of the invention.

In addition, this information gives rise to the possibility of gene therapy approaches to the treatment of the diseases. Treatment of the diseases using gene therapy approaches either involving somatic or germ line treatments utilising the sequence information provided herein forms a further aspect of the invention.

For example in somatic treatment, the mutant gene can be effectively "switched off" by administration of an appropriate anti-sense mRNA construct and these constructs form a further aspect of the invention. One can also insert a copy the correct gene using known techniques.

Therefore the invention provides a method of treating a disease selected from diabetes mellitus including non-insulin-dependent diabetes; maturity-onset-diabetes of the young and insulin-dependent diabetes; diseases which result in increased insulin secretion such as persistent hyperinsulinaemia of infancy; hypertension and hypotension; cerebral ischaemia; stroke; cerebral vasospasm; peripheral arterial disease; vascular smooth muscle

disease; angina pectoris; long QT syndrome; vasospastic angina; cardiac dysrhythmia; ischaemia induced cardiac arrhythmias; Parkinson's Disease; diseases associated with abnormal cerebellar function (eg ataxia) asthma; diseases of intestinal smooth muscle, including irritable bowel syndrome, urinary incontinence; male alopecia; certain skeletal muscle myopathies, such as those associated with membrane depolarisation or hyperpolarisation or hypopolarisation; appetite (both reduced and excessive); abnormalities and diseases of uterine smooth muscle, including premature labour; diseases involving abnormal nerve conduction; inappropriate secretion of renin and insensitivity to certain classes of drugs (eg sulphonylureas, K-channel openers etc) caused by inappropriate coupling between Kir6.2 and the drug-binding subunits of the channel complex, which comprises administration of a therapeutic agent which modifies the activity of the Kir6.2/SUR complex.

Experiments relating to the invention are now described by way of example only with reference to the drawings referred to below.

Figure 1 is the nucleotide sequence of the murine Kir6.2 gene and the amino acid sequence ascribed to it.

Figure 2 shows the predicted amino acid sequence of Kir6.2 and comparison with other members of the inward rectifier family. Residues shared with at least Kir6.1 are boxed; putative transmembrane (TM) and pore (H5) domains are overlined. Dashes represent minimal gaps introduced to maximise the comparison.

Figure 3 shows the tissue distribution of Kir6.2 Left: Poly A+ RNA (2μg/lane) from the specified tissues was hybridised with Kir6.2 Right. Total RNA (10μg/lane) was extracted from MIN6 cells or RINm5F cells and hybridised with Kir6.2.

Figure 4 shows the results of electrophysiology experiments detailed hereinafter: In particular: A. shows whole-cell currents recorded from a HEK293 cell cotransfected with cDNA encoding Kir6.2 and SUR in response to alternate 20 mV depolarizing or hyperpolarizing pulses from a holding potential of -30 mV.
B and C. show whole cell currents (B) and the corresponding current-voltage (I-V) relationship (C) recorded from a HEK293 cell cotransfected with Kir6.2 and SUR, in response to a series of voltage steps from -150 to +130 mV from a holding potential of -30 mV.
D. Current-voltage (I-V) relationships recorded for the same cell in 40 mM K (o), 20 mM K (■) and 5.6 mM K (•) solution. Holding potential, -30 mV. E. Mean relationship between the current reversal potential and $[K]_o$, for 6 cells. The line has a slope of 45 mV per decade change in $[K]_o$.

Figure 5 shows the results of electrophysiology experiments detailed hereinafter : In particular: Ai. shows whole-cell currents recorded before (left), during (centre) and after (right) addition of 5 mM barium to the external solution, from a HEK293 cell transfected with Kir 6.2 + SUR. Aii. I-V relationships recorded for the same cell in control solution (o, •), or in the presence of 5 mM barium (□).
Bi. shows whole-cell currents recorded before (left), during (centre) and after (right) addition of 0.5 mM tolbutamide to the external solution, from a HEK293 cell transfected

with Kir 6.2 +SUR. Bii. Current-voltage (I-V) relationships recorded for the same cell in control solution (o, •), or in the presence of 500 μM tolbutamide (□).
C. shows the relationship between tolbutamide concentration and the whole-cell current, expressed as a fraction of its amplitude in the absence of the drug, for a cell cotransfected with Kir 6.2 + SUR. The line is the best fit of a modified Hill equation to the data, using least squares analysis, and gives K_d = 2.4, n=1.04 and O = 0.2.
D. Mean whole-cell Kir6.2/SUR currents recorded at -100 mV in control solution (C) and in the presence of 0.5 mM tolbutamide (T), 5 mM barium (B), or 20 μM quinine (Q). The number of cells is indicated above the bars.

Figure 6 A, B. Single-channel currents (A) and corresponding current-voltage (i-v) relationship (B) recorded from an inside-out patch on an HEK293 cell cotransfected with Kir6.2 plus SUR. A. The dashed lines indicate the zero current level. The holding potential is indicated to the right of each trace. Filter frequency, 0.5 kHz; sample frequency, 1 kHz. B. The single-channel conductance was 71 pS (measured between -10mV and -70 mV).
C,D. Single-channel currents recorded at -70mV from the same inside-out patch as in A.1mM ATP (C) or 0.5 mM tolbutamide (D) were applied as indicated by the bars. Channel activity was reduced by 90% (C) and 97% (D). The dashed lines indicate the zero current level.

Figure 7 shows the nucleotide and amino acid sequence of the human Kir6.2. The boxed amino acids are those which are different to the amino acids at equivalent residues in the murine sequence as shown in Figure 1.

Figure 8 shows the putative topology of Kir6.2 showing the location of the mutations and polymorphisms identified in man.

Figure 9 shows effects of deleting residues in the C-terminus of Kir6.2 on K-ATP channel activity.

Figure 10 shows the effects of deleting residues in the C-terminus of Kir6.2 on the sensitivity of K-ATP channel activity to ATP

Figure 11 shows K-ATP channel activity following exposure to ATP for the wild-type K-ATP channel and the T224D mutation.

Figure 12 shows 670 nucleotides of the human Kir6.2 gene promoter. The transcribed Kir6.2 gene sequence begins at position 671 (not shown).

Example 1
Cloning and Sequencing of Murine Kir6.2

A cDNA library was prepared from the mouse insulinoma cell line MIN6 using a ZAP Express ™ cDNA synthesis kit (Stratagene). The average insert length was 3kb and the number of independent clones was 10^6. This library (5 x 10^5 phages) was screened at low stringency using mixed radiolabeled probes consisting of the full length cDNA of Kir6.1 and Kir1.1a (hybridized at 37°C with 30% formamide and then washed at 0.3 x SSC,

0.1% SDS, 42°C for 1 hour) and 4 positively hybridizing clones were isolated. Each isolated clone was excised using a helper phage (ExAssist™) and recircularised to form a pBK-CMV vector containing the insert. Two independent clones were sequenced and found to be identical. The nucleotide sequence of the murine Kir6.2 gene and the amino acid sequence ascribed to it is shown in Fig.1. The nucleotide sequence predicts a protein of 390 amino acids (M_r = 43,561), sharing 74% identity with Kir6.1 (uK_{ATP}-1; Inagaki et al., supra), 48% with Kir1.1a (ROMK1 Ho et al., supra), 50% with Kir2.1 (IRK1 Kubo Y. et al., Nature (1993) 362, 127-133), 49% with Kir3.1 (GIRK1 Kubo Y. et al., Nature (1993) 364, 802-806), 50% with Kir3.2 (BIR1 Bond et al., FEBS Lett. (1995) 367, 61-66) and 49% with Kir3.4 (rcK-ATP Ashford et al., (1994) supra). The predicted amino acid sequence of murine Kir6.2 is compared with the sequences for other members of the inward rectifier family in Fig. 2.

The homology to Kir6.1 suggests that the new clone belongs to the subfamily comprising Kir6.1 and should be named Kir6.2. In particular, like Kir6.1, a GFG motif is found in the putative pore-forming loop (underlined) instead of the GYG motif found in other Kir channels. A hydrophobicity plot of Kir6.2 reveal two putative transmembrane domains, as found for other Kir channels. There are two potential cAMP-dependent phosphorylation sites (Thr-224 and Ser-372), 6 potential protein kinase C-dependent phosphorylation sites (Ser-3, Ser-37, Thr-190, Thr-336, Thr-224 and Ser-363) and 3 potential casein kinase II-dependent phosphorylation sites (Thr-62, Thr-224 and Ser-354). There is no obvious nucleotide consensus sequence for ATP-binding.

Example 2
Tissue distribution experiment

Total RNA was isolated from MIN6 and RINm5F (rat insulinoma) cells using TRI-REAGENT (Molecular Research Centre) and 10μg of each total RNA, was separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham) and hybridised with Kir6.2 cDNA (entire coding region). The inventors also probed a Multiple Tissue Northern Blot (Clonetech) containing 2 μg poly A^+ RNA per lane from various tissues with Kir6.2 cDNA. The blots were washed at high stringency (0.1 x SSC, 65°C for 1-2 hours) and autoradiography was carried out for 2 days with intensifying screens.

The tissue distribution of Kir6.2 mRNA is illustrated in the Fig. 3 Northern blot analysis which shows two transcripts of 2.9 and 4.0 kilobases. These were strongly expressed in skeletal muscle, heart, brain and the insulinoma cells MIN6 and RINm5F. Weak expression was found in lung, very weak expression in kidney and no hybridisation was detected in spleen, liver or testis.

Example 3
Electrophysiology studies

HEK293 cells were transiently transfected with the pBK-CMV vector containing the coding sequence of Kir6.2 and/or SUR1 (subcloned into pcDNA3), using Lipofectin (Life-Technologies), as described by the manufacturer. In most experiments, the cells

were cotransfected with Kir6.2 and SUR1. In cotransfections the total DNA concentration was kept constant and the ratio of Kir6.2 to SUR1 was either 1:1 or 1:4. Mock-transfected cells received the same amount of plasmid without coding sequences, but were otherwise treated exactly the same.

Cells were assumed to express Kir6.2 if their currents were larger than 1.96 standard deviations above the mean current (95% confidence limits) observed in mock-transfected cells in parallel transfections. For Kir6.2 + SUR1 transfected cells, this amounted to 23 out of 38 cells dialysed with 0.3 mM ATP and 5/8 cells dialysed with 5 mM ATP: only these cells were used for analysis.

Cells were cultured before and after transfection in MEM medium, supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%). Whole-cell and single-channel currents were studied for 48-72 hour after transfection using the patch clamp technique. For whole-cell recordings, the pipette solution contained (mM): 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 HEPES (pH 7.2 with KOH; total K ~ 137 mM) and 0.3 mM ATP (or 5 mM ATP where indicated). The bath solution contained (mM): 40 KCl, 100 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4). Different K concentrations were obtained by equimolar substitution with NaCl. The holding potential was -30 mV. For inside-out patch recordings, the pipette, contained (mM): 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4) and the bath contained (mM): 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 HEPES (pH 7.2 with KOH; total K ~ 137 mM) plus MgATP and K_2ADP as indicated. Tolbutamide was dissolved in DMSO (final concentration <0.1%). Experiments were carried out at 22-25°C.

Drugs were tested once the whole-cell currents had reached a steady-state level (> 15 min), with 0.3 mM $[ATP]_i$. Tolbutamide dose-response curves were obtained for each cell at all drug concentrations. Test solutions were alternated with control solutions and the current (I) is plotted as a fraction of the mean (I_c) of that obtained in control solution before and after exposure to the drug. Test solutions were applied in random order. Dose-response curves were fitted to a modified form of the Hill equation: $I/Ic = 0 + ((1-0) / (1 + ([Tolb] / K_d)^n))$ where [Tolb] is the tolbutamide concentration, K_d is the tolbutamide concentration at which inhibition is half maximal, n is the Hill coefficient and 0 is an offset which describes the fact that in some cells the maximum block was not zero. Data are presented as mean ± SEM and vertical lines indicate one SEM. Statistical significance was tested using the paired t-test.

Single-channel currents recordings were filtered at 0.5 kHz or 2.5kHz (for kinetics) by an 8-pole Bessel filter, and sampled at 1 or 10 kHz using a Digidata A/D converter (Axon Instruments). Channel activity was calculated as the mean current amplitude divided by the single-channel current for data stretches of 1-2 min. Mean open and closed times were obtained by fitting the lifetime distributions using a Simplex maximum likelihood method.

The results of these experiments are shown in Figures 4, 5 and 6 hereinafter. No difference was observed in the amplitude of whole-cell currents recorded in mock-transfected and Kir6.2 transfected cells, which were -249 ± 32 pA (n=63) compared to -

262 ± 66 pA (n=25), respectively, at -100mV. This suggests Kir6.2 does not express functional channel activity when it is transfected by itself in HEK293 cells. In cells transfected with SUR1 alone the current amplitude was -291 ± 53 pA (n=41) at -100mV, which is not significantly different from mock-transfected cells and confirms that SUR1 does not itself form an ion channel. When cells were cotransfected with Kir6.2 and SUR1, however, whole-cell currents were initially larger than in mock-transfected cells (-743 ± 165 pA) and further increased to -2621 ± 460 pA (n=12), $p<0.001$), at -100mV, within the next 15 min (Fig. 3A). (A similar effect is found in pancreatic β-cells where it has been attributed to the washout of ATP from the cell following dialysis with the pipette solution (Trube et al., Pflüges Arch. (1986) 407, 493-499). No such increase in current was observed over a 15 min period in mock-transfected cells, Kir6.2-transfected cells or SUR1-transfected cells. Similarly no increase in current was observed when cells transfected with Kir6.2 + SUR1 were dialysed with 5 mM ATP, current amplitudes at -100 mV being -659 ± 56 pA at 0 min and -564 ± 105 pA at 10 min (n=5). This indicates that Kir6.2/SUR1 currents are inhibited by intracellular ATP.

Fig.4 B,C shows whole-cell currents and the associated current-voltage (I-V) relationship from a cell cotransfected with Kir6.2 and SUR1. Currents activated instantaneously upon hyperpolarization, were time-independent and showed weak inward rectification, properties which are characteristic of native K-ATP channels. The Kir6.2/SUR1 currents reversed at
-23.7 ± 1.4 mV (n=6) in 40 mM external K^+. Reduction of $[K]_0$ decreased the slope conductance and shifted the reversal potential to more negative potentials (Fig.4 D). The shift in the reversal potential was 45.2 ± 3.2 mV (n=6) for a 10-fold change in $[K]_0$ (Fig. 4E), indicating that the channel is highly K-selective.

The pharmacology of Kir6.2/SUR1 currents was consistent with that of native K-ATP channels. Thus, 5mM barium blocked the current in a time and voltage-dependent manner, the current being blocked by 74.9 ± 10.6 % (n=7, $p<0.01$) at -100 mV (Fig. 5A). Quinine (20 μM) inhibited Kir6.2/SUR currents by 68.9 ± 10.9% (n=5, $p<0.02$) at -100 mV (Fig. 5D), a potency consistent with that of native K-ATP channels (Bokvist K., et al., J. Physiol. (1990) 423, 327-342). Tolbutamide (0.5 mM) also blocked Kir6.2/SUR currents (Fig. 5B), by 68.7 ± 7.9% at -100 mV (n=14, $p<0.01$; Fig. 5D). A representative dose-response curve for tolbutamide inhibition is shown in Fig. 5C and was fitted best with a K_d =2.4 μM and a Hill coefficient of 1.04: mean values were 4.2 ± 1.4 and 0.91 ± 0.21 (n=5), respectively. These values are similar to those found for K-ATP currents in pancreatic β-cells (Ashcroft FM., Ashcroft FM et al., Trube et al., supra).

Fig. 6A shows single-channel currents recorded from an inside-out patch on a HEK293 cell cotransfected with Kir6.2 and SUR1. At negative potentials, channel openings were clustered into bursts separated by long closed periods, whereas openings of longer duration were found for outward currents. During the burst, the mean open and closed times were 1.51 ± 0.27 ms and 0.38 ± 0.05 ms (n=6) at -70 mV, respectively. There was no clear dependence of the open probability on membrane potential. The current-voltage relationship (Fig. 6B) showed weak inward rectification, the currents at +30 mV being 53 ± 7% (n=5) of those at -30 mV, and the mean single-channel conductance, measured over the linear part of the I-V relationship (from -10 to -70 mV), was 72.5 ±

2.2 pS (n=8). In inside-out patches, single-channel currents were inhibited by application of 1-5 mM ATP (Fig.6C and 3 others) or 0.5 mM tolbutamide (Fig. 6D). All these properties are characteristic of native K-ATP channels.

Single-channel currents were observed in 19/35 inside-out patches and at a higher frequency in cell-attached patches (>80% of patches in some transfections). In most cases more than one channel was found in each patch. A channel with similar kinetics and conductance (67.8 $\pm$ 0.8 pS, n=6) was observed occasionally in patches on mock-transfected cells (15/94 cell-attached patches; one channel per patch) but ran down rapidly after excision and was not inhibited by 1 mM ATP (6/6 patches).

Example 4
Cloning and sequencing of human Kir6.2

The human Kir6.2 gene was isolated from a human genomic library (Clonetech) using the full length mouse Kir6.2 cDNA as a probe. Screening of 10^6 plaques was carried out using 30% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA at 37°C. The filters were washed with 0.2 x SSC at room temperature. Clones were purified by screening at reduced density. DNA was prepared from a positively-hybridising clone and an EcoRI-SalI fragment containing Kir6.2 was subcloned and sequenced.

The predicted amino acid sequence for mouse and human Kir6.2 are highly homologous. Only 14 amino acids out of a total of 390 amino acids differ between the two sequences ie greater than 95% homologous at the amino acid level. The entire coding sequence of the human gene was contained within a single exon.

Example 5
Chromosomal localisation of human Kir6.2

The chromosomal location of Kir6.2 was determined by fluorescent *in situ* hybridisation (FISH). Total phage DNA was labelled with biotin-11-dUP by nick translation. The hybridisation mixture contained 200 ng labelled probe DNA and 2.5 μg unlabelled human Cot-1 DNA, previously denatured and annealed at 37°C for 15 min prior to hybridisation. Hybridisation to normal human male metaphase chromosomes was carried out at 42°C overnight. After stringent washes, the site of hybridisation was detected with successive layers of fluorescin-conjugated avidin (5 μg/ml, Vector labs) and biotinylated with anti-avidin (5 μg/ml, Vector Labs). Slides were mounted in Vectashield (Vector Labs) containing antifade, 1μg/ml propidium iodide and 1 μg/ml 4'6' diamidinophenylindole (DAPI) to allow concurrent G-banded analysis under UV light. Results were analysed and images captured using a Bio-Rad MRC 600 confocal laser scanning microscope.

The human geneKir6.2 gene was localised to chromosome 11p15.1. It is located adjacent to the SUR1 gene (Thomas et al., (1995) Science 268, 425-429).

Example 6
Identification of polymorphisms in the human Kir6.2 gene.

Genomic DNA was obtained from peripheral leukocytes from 90 normal humans and from 135 consecutive white Caucasian and 39 Afro-Caribbean patients with non-insulin-dependent diabetes mellitus (NIDDM). The coding region of the Kir6.2 gene is continued within a single exon and PCR primers were chosen to amplify this Kir6.2 containing exon and the adjacent flanking regions.

Sense (antisense) strand:

Segment 1.	5'GGTGCCTCCGATGGGGGAAG3'	(AAGGACATGGTGAAGATGAG)
Segment 2.	GACCTCAAGTGGCCACACAC	(TCCTCAGTCACCATGCGCCC)
Segment 3.	CTTCCTTTTCTCCATTGAGG	(ATCATGCTCTTGCGGAGGTC)
Segment 4.	CCGCCTCTGCTTCATGCTAC	(ACCACGCCTTCCAGGATGAC)
Segment 5.	CCTGCACCACCACCAGGACC	(GTGTGGGCACTTTGATGGTG)
Segment 6.	GTGGACTACTCCAAGTTTGG	(GGCTACATACCACATGGTCC)

The amplified DNA fragments were designed to overlap one another and to be smaller than 300 base pairs in order to maximise the possibility of detecting genetic variations by SSCP. SSCP under two different conditions (the presence or absence of 10% glycerol) was carried out by the method as disclosed in Sakura et al.1995 (FEBS Lett 367:193-197) except for annealing temperatures of 55°C (segments 2,3,4 and 5) or 60°C (segments 1 and 6). When a polymorphism was identified, the PCR fragment was subcloned and sequenced. More than two independent clones were sequenced for each polymorphism.

In the white Caucasians, six polymorphisms were detected. Fig 9 shows a putative topology of Kir6.2 showing the location of the mutations and polymorphisms identified. All of the mutations identified were found in the cytosolic domains of the channel, with E23K being located in the N-terminal tail and the others in the C-terminal tail. Direct sequence nucleotide analysis showed one of the polymorphisms to be a silent mutation, A190A (GCT to GCC). Four other mutations resulted in amino acid changes, these being E23K (GAG toAAG), L270V (CTG to GTG), I337V (ATC to GTC) and S385C (TCT to TGT). In addition, the inventors detected an insertion of two amino acids, KP (AAGCCC), after position 380 in one diabetic individual. In all cases where the full length Kir6.2 DNA was sequenced (>10 individuals), the inventors found that the residue at position 148 was I (ATC), not S (AGC) as previously reported. The E23K and I337V variants were found to be completely linked: ie an individual having E at position 23 would also have I at position 337. The variant L270V was found to be associated with higher fasting insulin concentrations and thus it is a candidate for affecting insulin action in muscle, in which Kir6.2 is expressed.

One of the Afro-Carribeans had two new mutations L355P (CTA to CCA) and S363S (silent TCA to TCG), but otherwise the same polymorphisms were identified as in the white Caucasians.

Example 7
The brain K-ATP channel has Kir 6.2 and SUR1 subunits.

In-situ-hybridization analysis was performed on adult rat and mouse brain sections using 45-50 base pair synthetic antisense and sense oligonucleotides from regions in the open reading frame or untranslated region showing least similarity to other subfamily members to minimize cross-hybridization. The high specificity and effectiveness of the probes as

well as their background labelling was tested as described for Kir2.0 and Kir3.0 subfamily members in Karschin, C., et al., 1996 J. Neurosci. 16,3559-3570; controls used sense oligonucleotides in adjacent sections, RNAase digestion before hybridization and hybridization with a mixed oligonucleotide probe containing a 10X excess of cold probe. The sequence and location (base pair on coding stand indicated) of the oligonucleotides used for the data analysis were as follows below.

Kir6.2 3' ORF antisense 1301
5'GGACAAGGAATCCGGAGAGATGCTAAACTTGGGCT TGGCCTTTGC3' (mouse with two mismatches to the rat ortholog),
Kir6.2 3' UTR +85
5'GTCTGGCCAAGAGGCTCGCACCCCACCACTCTACATACCAT ACTT3' (mouse with no specific match to the rat ortholog)
SUR1 ORF antisense 2164 5'AGCGAGGACTTGCCACAGCCCACCTGCCCCACGAT CATGGTCAGC3'
SUR1 3' ORF/UTR antisense +35 5'
GGTGAGGTGTGGGGTGGCACTTTGGCGCTGG
CTGGTCATTGTC

Oligonucleotides were 3' end labelled with [35S] dATP (Dupont/New England Nuclear,1200 Ci/mmol) by terminal deoxynucleotidyl transferase (TdT, Boehringer Mannheim, Germany) and used for hybridization at concentrations of 2-10pg/μl (400000cpm/100μl hybridization buffer per slide). For tissue preparation, adult Wistar rats and adult NMRI mice were anaesthetized, decapitated and the brain removed and quickly frozen on dry ice. Brains were cut on a cryostat at 10-16 μm, thaw mounted onto silane-coated slides, fixed with 4% paraformaldehyde in PBS (pH7.4), dehydrated and stored under ethanol until hybridization. Slides were air dried and hybridized overnight at 43°C in 100μl buffer containing 50% formamide, 10% dextran sulfate, 50mM DTT, 0.3M NaCl, 30mM Tris-HCl, 4mM EDTA,1 x Denhardts solution, 0.5mg/ml denatured salmon sperm DNA and 0.5mg/ml polyadenylic acid. Sections were washed 2 x 30 min in 1 x SSC plus 50mM beta-mercaptoethanol, 1 hour in 1 x SSC at 60°C and 10 min in 0.1 x SSC at room temperature. Specimens were then dehydrated, air-dried and exposed to Kodak BIOMAX x-ray film for 8-34 days. For cellular resolution, selected slides were subsequently dipped in photographic emulsion Kodak NTB2, incubated for 8-16 weeks and then developed in Kodak D-19 for 2.5 min. For analysis with bright and darkfold optics, sections were Nissel counterstained with cresyl violet to confirm cytoarchitecture. Brain structures were identified and confirmed according to Paxinos and Watson ("The Rat Brain in Stereotaxic Coordinates", Academic Press, Sydney).

Briefly, the expression patterns of Kir6.2 and SUR1 mRNAs were widely overlapping and pronounced hybridization signals were observed for both mRNAs in the majority of brain regions. Compared to the expression patterns of other Kir subfamily members, the expression of both transcripts was more widespread and diffuse and thus more difficult to analyse. Peak expression levels were in the hippocampus, neocortex, olfactory bub, cerebellum and several distinct nuclei of the midbrain and brainstem, indicating their important role in vital brain function. Full results are shown in Table 1. For analysis of emulsion-dipped slides, silver grain density over the most intensely labelled cell bodies was rated as very strong (++++), and other brain regions were rated in relation to this

maximal signal as strong (+++), moderate (++), low (+) or background level (0). The use of (nd) indicates that expression could not be determined. Kir6.2 mRNA expression was determined in mouse brain and SUR1 in rat brain sections.

Example 8
Xenopus oocyte as an expression system for cloned K-ATP channels

Female Xenopus laevis were anaesthetized with MS222 (2g/l added to the water). One ovary was removed via a mini-laparotomy, the incision sutured and the animal allowed to recover. Immature stage V-VI Xenpous oocytes were incubated for 75 min with 1.5mg/ml collagenase (Boehringer, type A) and manually defollicated. Oocytes were then coinjected with a single type of mRNA or a mixture of mRNAs.

Kir6.2 (Genbank accession number D50581); was cloned from a mouse insulinoma cDNA library as discussed above. SUR1 was cloned from hamster insulinoma (HIT-T15) cells (Genbank accession number L40623).

Kir6.2 was cloned in the pBK-CMV vector (Sakura, H. et al., 1995 FEBS Letters 377,338-344) and SUR1 in the vector pcDNA3. Capped mRNA was synthesized by in vitro transcription from linearized cDNA and stored in 10mM tris HCl,pH 7.4 at -80C.

About equal amounts (~25ng) of the different mRNAs were mixed prior to injection. Control oocytes were injected with 10mM Tris HCl. The final injection volume was ~50nl per oocyte. Isolated oocytes were maintained in modified Barth's solution containing (in mM): 88 NaCl, 1 KCl, 1.7 $MgSO_4$, 0.47 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 2.4 $NaHCO_3$ 10 HEPES (pH7.4), supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 5mM pyruvate.

Oocytes injected in this way exhibit large inwardly rectifying K-currents when cytosolic ATP levels are lowered by the metabolic inhibitors azide or FCCP. No currents were observed in response to azide in oocytes injected with either Kir6.2 or SUR1 alone, indicating that both are needed for functional activity. The pharmacological properties of Kir6.2/SUR1 channels expressed in oocytes resembled those of native K-ATP channels eg >90% blocked by tolbutamide (500μM) meglitinide (10μM) and glibenclamide (100nM) and 50% blocked by 5μM tolbutamide. They were also activated 1.8 fold by diazoxide (340μM), 1.4-fold by pinacidil (1mM) and unaffected by etomakalim (0.5mM).

Example 9
Region of Kir6.2 important for coupling to SUR1

The inventors have identified one region of Kir6.2 which appears to be important for coupling to SUR1. This is the C-terminal region of the protein. As shown in Fig. 10, deletion of the last 35 residues of Kir6.2 (365-390 (end)), significantly reduces the ability of 100μM diazoxide (in the presence of 100μM MgATP) to potentiate channel activity. The ability of MgADP (100μM) to enhance channel activity likewise reduced, as is the inhibitory effect of 500μM tolbutamide. The sensitivity of channel activity to inhibition by ATP was increased, the Ki being 19.6$\pm$2.5μM (n=x) for the deleted Kir6.2 as compared with 31.5$\pm$0.4μM (n=x) for the wild-type channel (see Fig. 11). These data

argue that the C-terminal end of Kir6.2 is involved in coupling SUR1 to K-ATP channel activity. As stated in the original patent application, drugs designed to enhance or reduce this interaction may be of therapeutic benefit. These new data identify one site of interaction.

Example 10
Reversed of 'rundown' by exposure to MgATP : mutation of threonine residue in a putative consensus sequence of protein kinase A phosphorylation in Kir6.2 associated with markedly enhanced rundown of channel activity

K-ATP channel activity declines with time following patch excision, a phenomenon known as rundown. This rundown of channel activity is reversed by exposure of MgATP. As shown in Fig. 12 channel activity following exposure to MgATP (which blocks the channel) was greater than that recorded in the control solution prior to application of the nucleotide. This argues that channel activity may be regulated by phosphorylation. We have identified a putative consensus sequence of protein kinase A phosphorylation in Kir6.2. Mutation of a threonine residue within this consensus sequence (threonine 224) influences the rate of channel rundown. When this threonine is mutated to an aspartate residue (T224D), the mutant channels show a markedly enhanced rundown of channel activity (Fig. 3). The T224D mutation mimics permeant phosphorylation of the channel. Mutation to an alanine (which is predicted to prevent phosphorylation) may be expected to prevent rundown substantially altogether. K-ATP channels carrying this mutation may be of particular use for drug screening purposes.

Example 11
Cloning and Sequencing the human Kir6.2 promoter

A human genomic library (Clontech) was screened with the entire mouse Kir6.2 gene. Two independent positive clones were obtained, and a 3.5kb subfragment containing the 6.2 gene and flanking DNA was isolated from both. This fragment was sequenced using a primer complementary to the 5' end of the 6.2 gene, to give the 670nts shown in Fig. 12. That this sequence is indeed the Kir6.2 promoter is supported by the following facts:
a) It lies immediately 5' of the 6.2 gene in a single genomic DNA fragment, and the same sequence was obtained from 2 independent clones.
b) The sequence contains multiple consensus sites for transcription factors (sp1, AP-2 and several others, identified by two separate datebase searches).
(c) The sequence is highly homologous to several other mammalian promoters, for example snRNP E (over 80% identical in a 189bp region).

TABLE 1: Distribution of Kir 6.2/SUR1 mRNAs in the adult mouse and rat brain

Brain region	Kir6.2	SUR1
Olfactory bulb		
granule cells	+	+
mitral cells	+++	++
periglomerular cells	++	++
Anterior olfactory nucleus	++	+++
Olfactory tubercle	++	++
Piriform cortex	+++	+++
Neocortex	+++	+++
Subiculum	++	+++
Entorhinal cortex	+++	+++
Hippocampus		
Dentate gyrus granule cells	+++	+++
CA1, CA3 pyramidal cells	++	++
CA2 pyramidal cells	++	++++
tenia tecta	+++	+++
indusium griseum	+++	+++
Septum		
Bed nuclei of stria terminalis	+	+
Lateral septal nucleus	+	+
Septohippocampal nucleus	+	++
Nuclei of the diagonal band	+++	++
Basal ganglia		
Caudate putamen	++	+
Globus pallidus	++	+
Ventral pallidum	+	+
Nucleus accumbens	++	0
Subthalamic nucleus	+++	+
Substantia nigra	++	++
Zona incerta	++	+
Amygdala	+	nd
Lateral olfactory tract nucleus	+++	++++
Hypothalamus	+	+
Preoptic area	+	+
Magnocellular preoptic nu.	+++	nd
Thalamus		
Thalamic reticular nucleus	+++	+
Geniculate nuclei	+	+
Anterior dorsal nucleus	+++	++
Lateral nuclei	+	+
Ventroposterior nuclei	++	++
Midbrain		
Superior colliculus	++	+
Inferior colliculus	++	++
Central gray	++	nd
Red nucleus	++	nd
Ventral tegmental area	++	+
Cerebellum		
Deep nuclei	+++	++

Molecular layer	+	+
Granule cell layer	+	++
Purkinje cells	+++	+
Brainstem		
Pontine nucleus	++	+++
Inferior olivary nuclei	+	0
Locus coeruleus	+	nd
Raphe nuclei	+	+
Pontine reticular formation	++	++
Spinal trigeminal nucleus	++	++
Facial nucleus	+	+++
Vestibular nucleus	+++	++
Cochlear nuclei	+++	++
hypoglossal nucleus	+++	++

CLAIMS

1. A nucleic acid molecule which has a nucleotide sequence encoding a protein which forms part or all of a K-ATP channel, which protein has part or all of an amino acid sequence as shown in Fig. 1 or Fig. 7.

2. A nucleic acid molecule according to claim 1 wherein the protein forms part of a K-ATP channel and that part comprises a subunit of a K-ATP channel.

3. A nucleic acid molecule according to claim 2 wherein the subunit forms a pore.

4. A nucleic acid molecule according to any one of claims 1 to 3 wherein the protein is able to complex with a SUR and the protein complexed with SUR has K-ATP channel activity.

5. A nucleic acid molecule according to any one of claims 1 to 4 wherein the nucleotide sequence is complementary to, or as shown in Fig. 1 or Fig. 7, or a mutant, variant, derivative, allele or fragment of a sequence as shown in Fig. 1 or Fig. 7 or of a sequence complementary to a sequence as shown in Fig. 1 or Fig. 7.

6. A nucleic acid molecule according to any one of claims 1 to 5 wherein the protein is specific to a K-ATP channel.

7. A nucleic acid molecule which has a nucleotide sequence which is able to function as a promoter for the expression of a subunit of a K-ATP channel.

8. A nucleic acid molecule according to claim 7 wherein the nucleotide sequence is complementary to, or as shown in Fig. 12; or a mutant, variant, derivative, allele or fragment of a sequence as shown in Fig. 12 or of a sequence complementary to a sequence as shown in Fig. 12 which retains functional activity as a promoter.

9. A replicable cloning or expression vector which comprises a nucleic acid molecule according to any one of claims 1 to 8.

10. A host cell which comprises a replicable cloning or expression vector according to claim 9.

11. A Xenopus oocyte which expresses a nucleic acid molecule according to any one of claims 1 to 8 or an expression vector according to claim 9.

12. A labelled hybridization probe which comprises a nucleotide sequence which comprises part or all of a nucleotide sequence as defined in any one of claims 1 to 8.

13. An oligonucleotide primer or an oligonucleotide primer pair for amplifying a nucleic acid molecule according to any one of claims 1 to 8.

14. Use of part or all of a nucleic acid molecule according to any one of claims 1 to 6 or a probe according to claim 12 or a primer or primer pair according to claim 13 to identify and obtain related nucleic acid molecules which encode proteins of K-ATP channels other than those having an amino acid sequence as shown in Fig. 1 or Fig. 7.

15. Use of part or all of a nucleic acid molecule according to claim 7 or claim 8 or a probe according to claim 12 or a primer or primer pair according to claim 13 to identify and obtain related nucleic acid molecules which encode a promoter for the expression of a subunit of a K-ATP channel.

16. A polypeptide which comprises part or all of a K-ATP channel.

17. A polypeptide according to claim 16 which forms part of an K-ATP channel and wherein said part comprises a pore-forming subunit of a K-ATP channel which is able to complex with a SUR and which when complexed, has K-ATP channel activity.

18. A polypeptide which has an amino acid sequence as shown in Fig. 1 or Fig. 7 or which is an amino acid sequence variant, allele, derivative, mutant or fragment of a polypeptide which has an amino acid sequence as shown in Fig. 1 or Fig. 7 and wherein a said variant, allele, derivative, mutant or fragment has characteristics of a K-ATP channel.

19. A variant, allelic, derivative or mutant polypeptide according to claim 18 which is able to complex with a SUR, the polypeptide complexed with SUR having K-ATP channel activity.

20. A binding member for a polypeptide according to any one of claims 16 to 19 which comprises an antibody binding domain specific for a said polypeptide.

21. A pharmaceutical composition which comprises either a nucleic acid molecule according to any one of claims 1 to 8, an expression vector according to claim 9 or a polypeptide according to any one of claims 16 to 19 in a therapeutically effective amount in combination with an appropriate physiologically acceptable carrier or excipient.

22. Use of a nucleic acid molecule according to any one of claims 1 to 8 or a polypeptide according to any one of claims 16 to 19 in a method for screening for a drug for treatment of a disease condition associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity.

23. Use of a nucleic acid molecule according to any one of claims 1 to 8, an expression vector according to claim 9 or a polypeptide according to any one of claims 16 to 19 to prepare a medicament for the treatment of a disease condition selected from diabetes mellitus including non-insulin-dependent diabetes; maturity-onset-diabetes of the young and insulin-dependent diabetes; diseases which result in increased insulin secretion such as persistent hyperinsulinaemia of infancy;

hypertension and hypotension; cerebral ischaemia; stroke; cerebral vasospasm; peripheral arterial disease; vascular smooth muscle disease; angina pectoris; long QT syndrome; vasospastic angina; cardiac dysrhythmia; ischaemia induced cardiac arrhythmias; Parkinson's Disease; diseases associated with abnormal cerebellar function (eg ataxia) asthma; diseases of intestinal smooth muscle, including irritable bowel syndrome; urinary incontinence; male alopecia; certain skeletal muscle myopathies, such as those associated with membrane depolarisation, hyperpolarisation or hypopolarisation; appetite (both reduced and excessive); abnormalities and diseases of uterine smooth muscle, including premature labour; diseases involving abnormal nerve conduction; inappropriate secretion of renin and insensitivity to certain classes of drugs (eg sulphonylureas, K-channel openers etc) caused by inappropriate coupling between Kir6.2 and the drug-binding subunits of the channel complex.

Fig 1

Mouse ATP-sensitive Potassium Channel Gene (Kir6.2)

nucleotides

GCCAAGCCAGTGTAGTGCCTCCCCCCATGGGGGAAACCCCTTCCCGGGGCCAACGGAGCC ↵

ATGCTGTCCCGAAAGGGCATTATCCCTGAGGAATATGTGCTGACCCGGCTGGCAGAGGAC
M L S R K G I I P E E Y V L T R L A E D ↵

amino acid

CCTGCAGAGCCCAGGTACCGTACTCGAGAGAGGAGGGCCCGCTTCGTGTCCAAGAAAGGC
P A E P R Y R T R E R R A R F V S K K G

AACTGCAACGTCGCCCACAAGAACATTCGAGAGCAGGGCCGCTTCCTGCAGGATGTGTTC
N C N V A H K N I R E Q G R F L Q D V F

ACCACGCTGGTGGACCTCAAATGGCCACACACTCTGCTCATTTTCACCATGTCCTTCCTG
T T L V D L K W P H T L L I F T M S F L

TGCAGCTGGCTGCTCTTTGCCATGGTCTGGTGGCTCATCGCCTTCGCCCACGGTGACCTG
C S W L L F A M V W W L I A F A H G D L

GCCCCCGGAGAGGGCACCAATGTGCCCTGCGTCACAAGCATCCACTCCTTTTCATCTGCC
A P G E G T N V P C V T S I H S F S S A

TTCCTTTTCTCCATCGAGGTCCAGGTGACCATTGGTTTCGGCGGGCGCATGGTGACAGAG
F L F S I E V Q V T I G F G G R M V T E

GAATGTCCCCTGGCCATCCTCATTCTCATTGTGCAGAATATCGTCGGGCTGATGATCAAC
E C P L A I L I L I V Q N I V G L M I N

GCCATCATGCTGGGCTGCATCTTCATGAAAACGGCCCAGGCCCATCGGCGGGCAGAAACC
A I M L G C I F M K T A Q A H R R A E T

CTCATCTTCAGCAAGCATGCTGTGATCACCCTGCGCCATGGCCGCCTGTGCTTCATGCTG
L I F S K H A V I T L R H G R L C F M L

CGCGTAGGGGACCTCCGAAAGAGCATGATCATTAGCGCCACCATCCACATGCAGGTGGTG
R V G D L R K S M I I S A T I H M Q V V

Fig 1 (cont)

CGCAAGACCACCAGCCCCGAGGGCGAAGTTGTGCCTCTCCACCAGGTAGACATCCCCATG
R K T T S P E G E V V P L H Q V D I P M

GAGAATGGCGTGGGTGGTAACGGCATCTTCCTGGTGGCCCCACTCATCATCTACCACGTC
E N G V G G N G I F L V A P L I I Y H V

ATCGACTCCAACAGCCCGCTCTACGACCTGGCTCCTAGTGACCTGCACCACCACCAGGAC
I D S N S P L Y D L A P S D L H H H Q D

CTGGAGATCATTGTCATCTTGGAAGGCGTGGTAGAAACCACGGGCATCACCACCCAGGCC
L E I I V I L E G V V E T T G I T T Q A

CGCACCTCCTACCTAGCTGACGAGATTCTATGGGGGCAGCGCTTTGTCCCCATTGTGGCC
R T S Y L A D E I L W G Q R F V P I V A

GAGGAGGACGGCCGCTATTCTGTGGACTACTCCAAATTTGGTAACACCATTAAAGTGCCC
E E D G R Y S V D Y S K F G N T I K V P

ACACCACTCTGCACAGCCCGCCAGCTTGATGAGGACCGCAGTCTGCTGGATGCCCTGACC
T P L C T A R Q L D E D R S L L D A L T

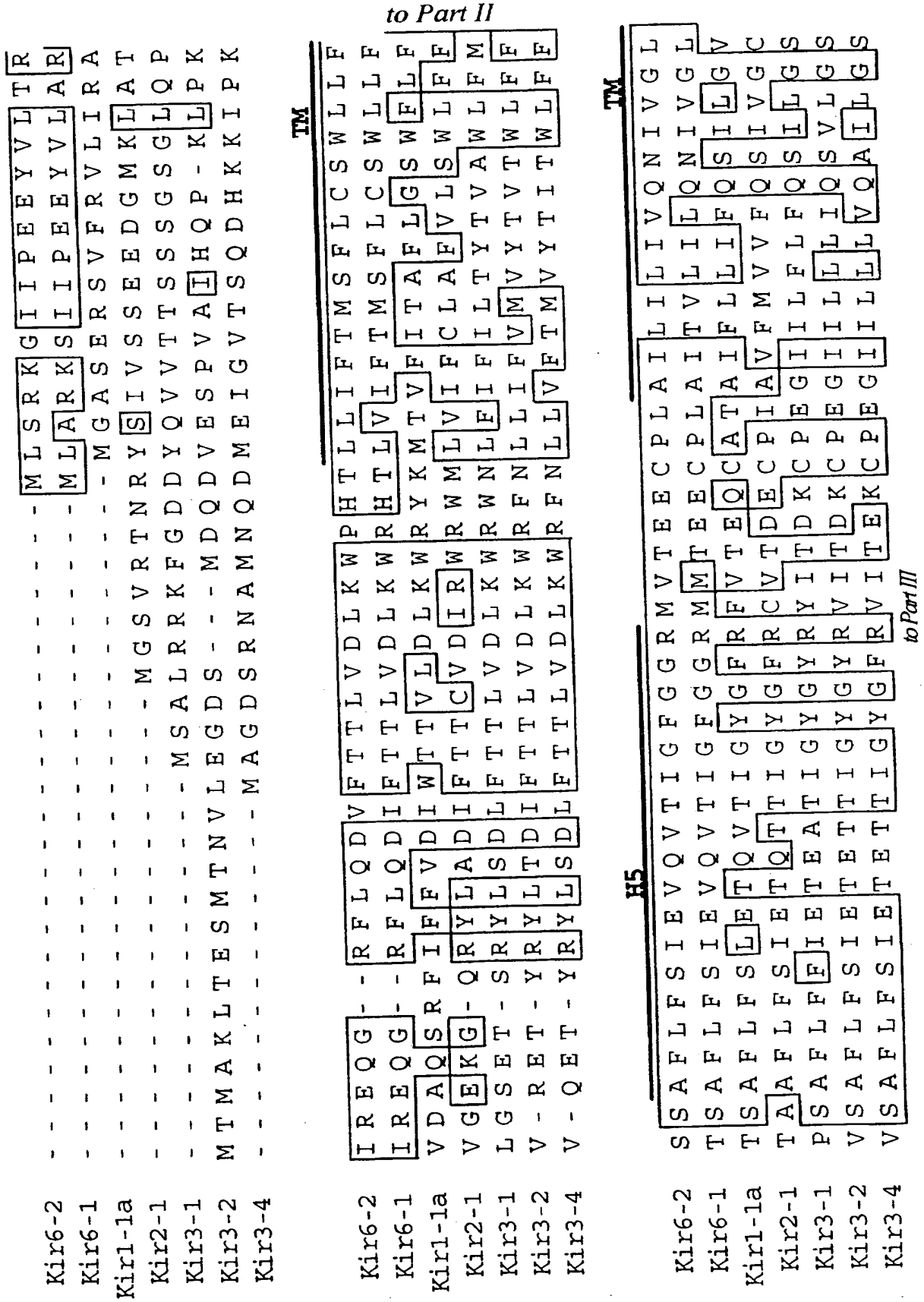
CTCGCCTCGTCGCGGGGGCCCCTGCGCAAGCGCAGTGTGGCTGTGGCGAAGGCCAAGCCC
L A S S R G P L R K R S V A V A K A K P

AAGTTTAGCATCTCTCCAGATTCCTTGTCCTGAGTTGCAGTTCCTCAGGCCCCCACTCAC
K F S I S P D S L S *

TTGTGTGGGCACGTGGAAAGTGAAGTATGGTATGTAGAGTGGTGGGGTGCGAGCCTCTTG

GCCAGACGAGGG

Fig 2 (Part I)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Kir3-4

Fig 2 (Part II)

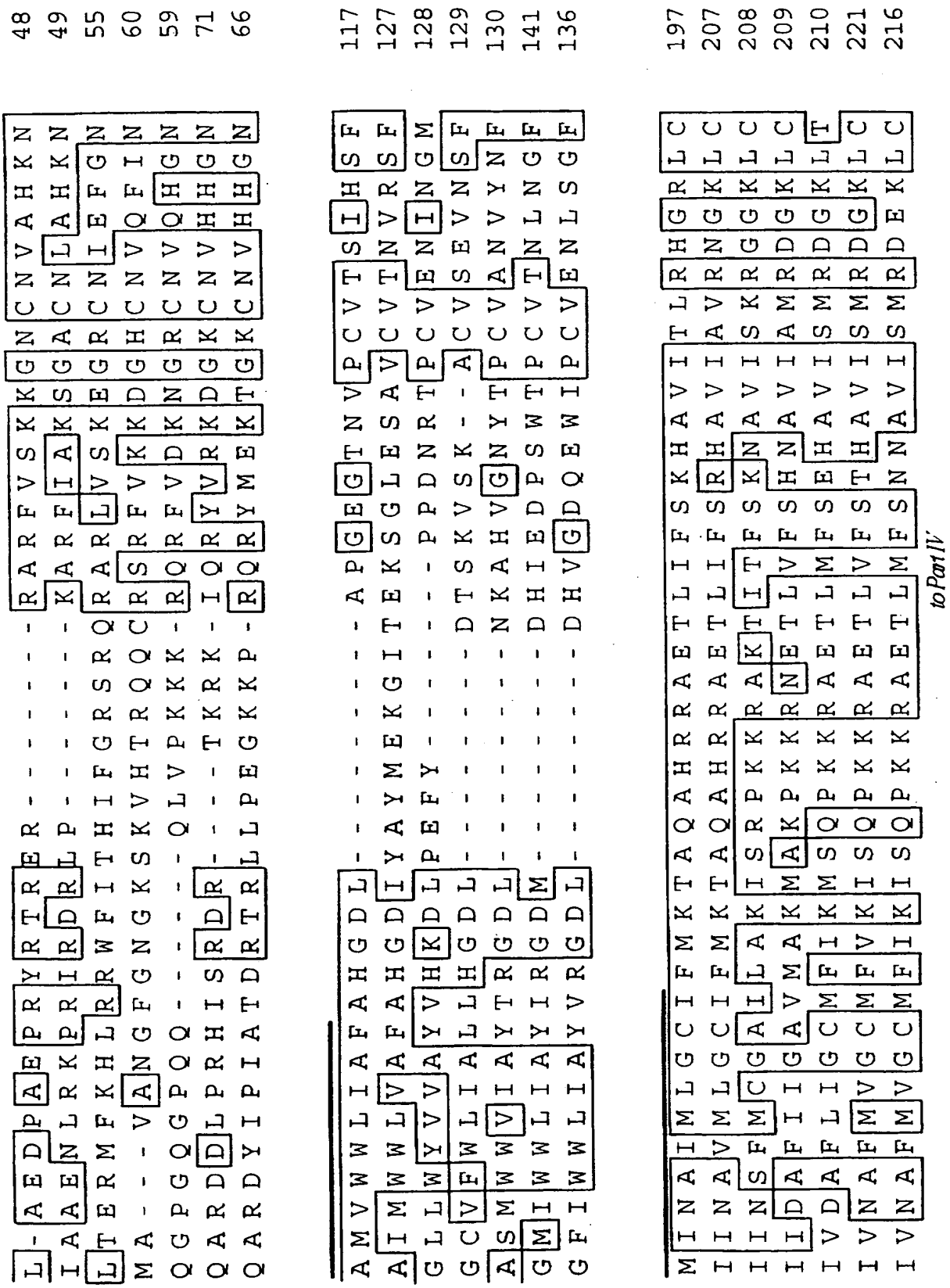
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Fig 2 (Part III)

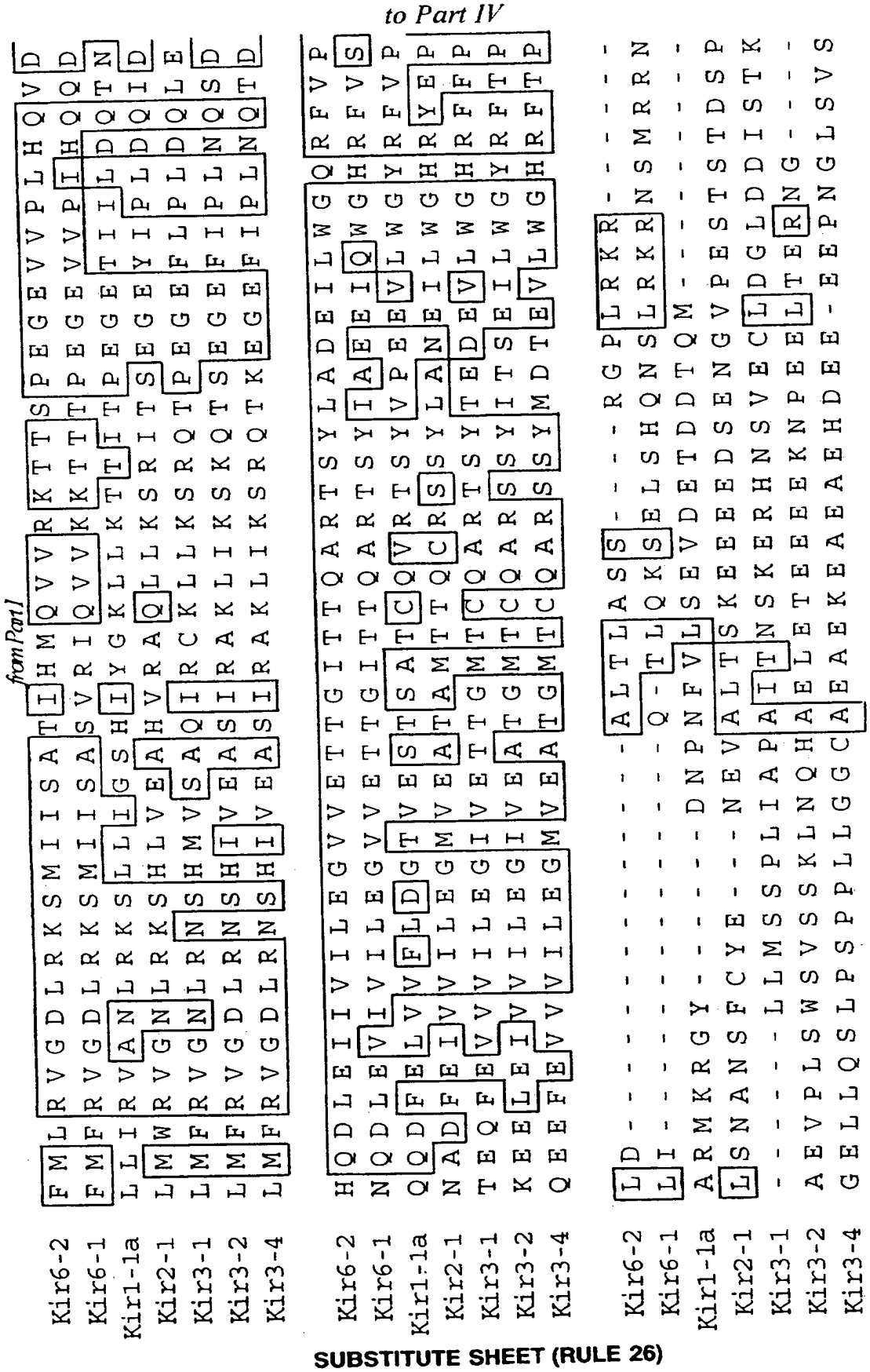

Fig 2 (Part IV)

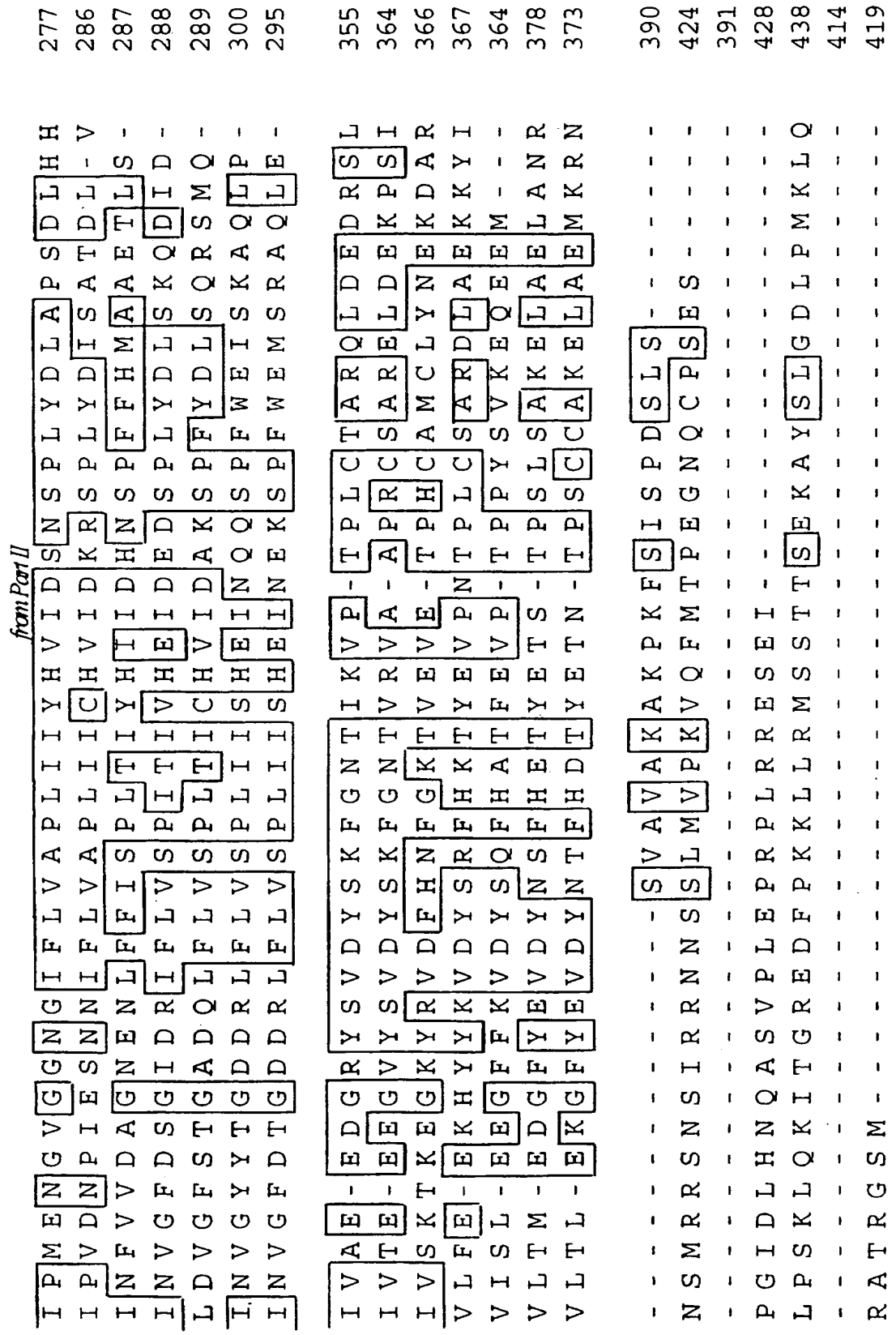
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Fig 3

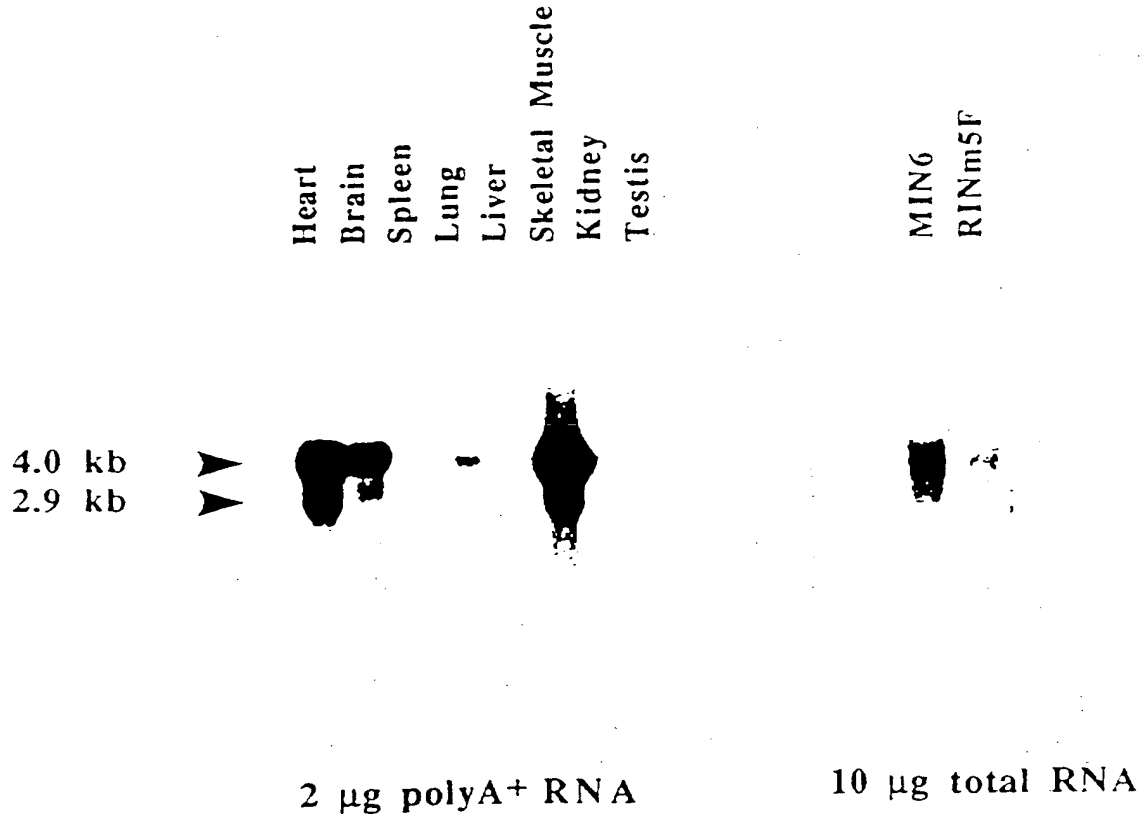
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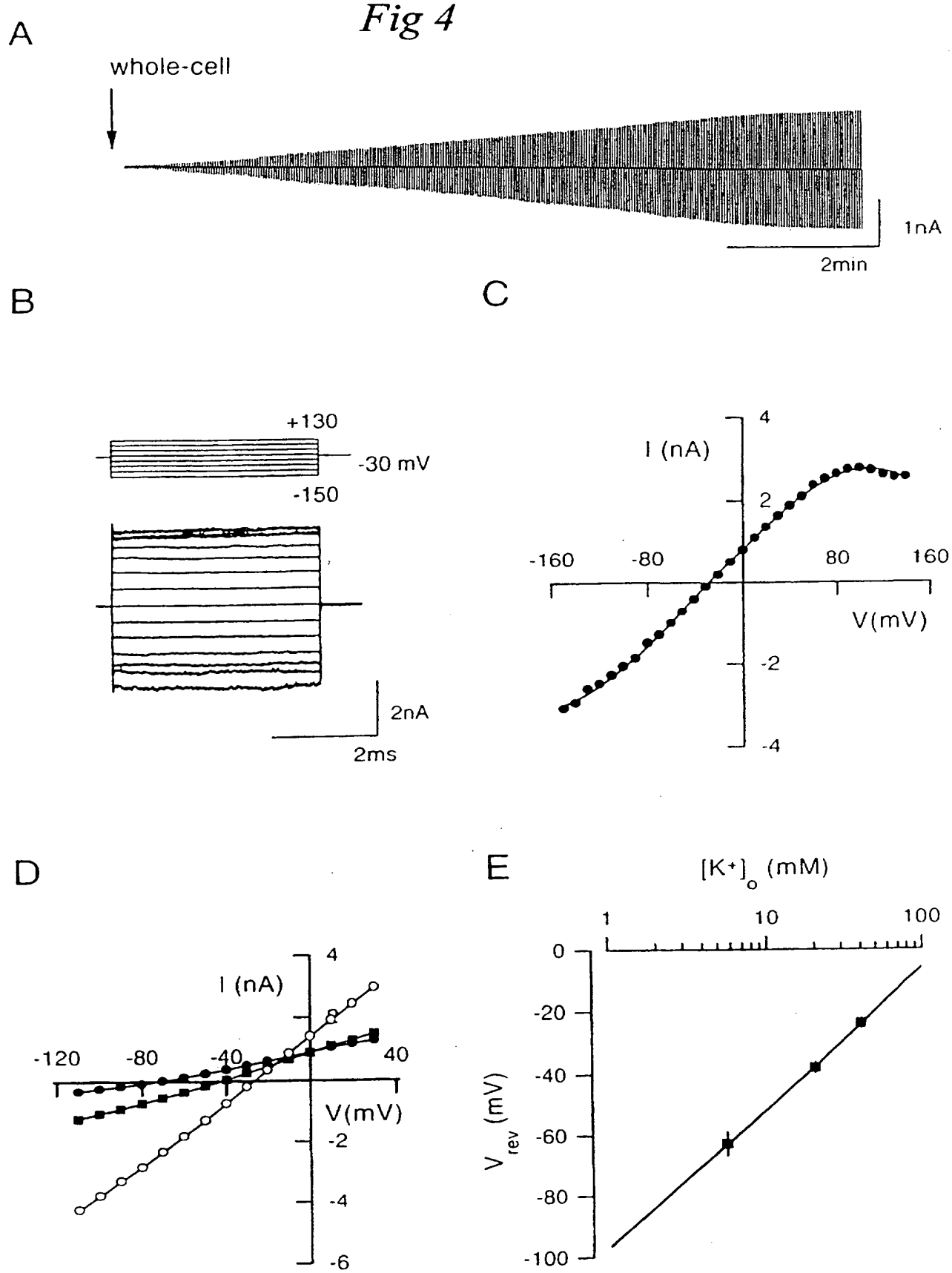
Fig 4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-160
-80
80
160
V(mV)
D
I (nA)
4
-2
-4
-6
-120
-80
-40
40
V(mV)
E
[K+]o (mM)
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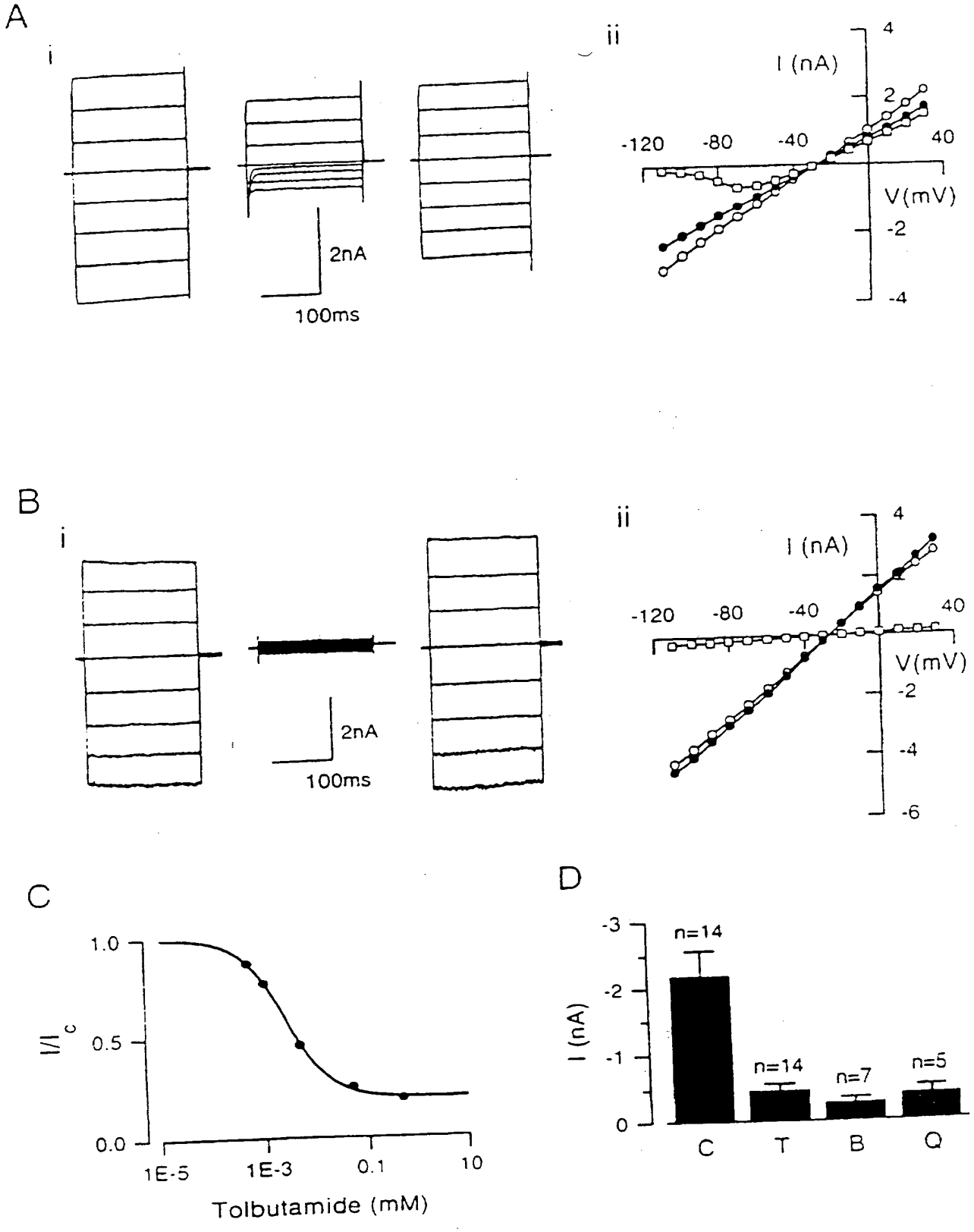
100
0
-20
-40
-60
-80
-100
Vrev (mV)

Fig 5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100ms
ii
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-4
-120
-80
-40
40
V(mV)
B
i
2nA
100ms
ii
I (nA)
4
-2
-4
-6
-120
-80
-40
40
V(mV)
C
I/I c
1.0
0.5
0.0
1E-5
1E-3
0.1
10
Tolbutamide (mM)
D
I (nA)
-3
-2
-1
0
n=14
n=14
n=7
n=5
C
T
B
Q

Fig 6

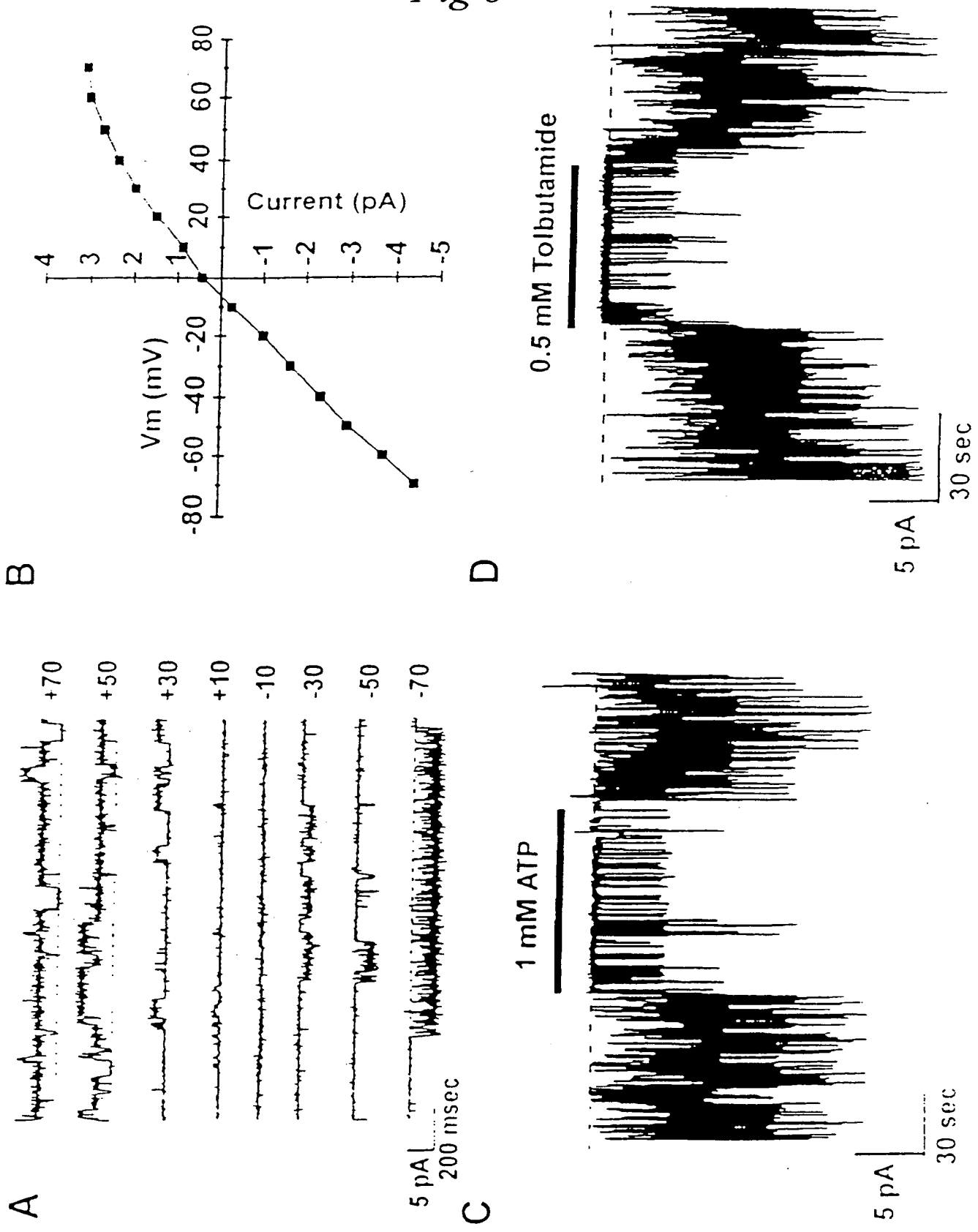
A
+70
+50
+30
+10
-10
-30
-50
-70
5 pA
200 msec
B
Current (pA)
Vm (mV)
4
3
2
1
-1
-2
-3
-4
-5
80
60
40
20
-20
-40
-60
-80
C
1 mM ATP
5 pA
30 sec
D
0.5 mM Tolbutamide
5 pA
30 sec

Fig 7

human Kir 6.2 gene

```
                                                GGTGCCTCCGATGGGGGAAGCCCCTCCCTGGGGGTCACCGGAGCC

ATGCTGTCCCGCAAGGGCATCATCCCCGAGGAATACGTGCTGACACGCCTGGCAGAGGAC
 M  L  S  R  K  G  I  I  P  E  E  Y  V  L  T  R  L  A  E  D

CCTGCCGAGCCCAGGTACCGTGCCCGCCAGCGGAGGGCCCGCTTTGTGTCCAAGAAAGGC
 P  A  E  P  R  Y  R [A] R [Q] R  R  A  R  F  V  S  K  K  G

AACTGCAACGTGGCCCACAAGAACATCCGGGAGCAGGGCCGCTTCCTGCAGGACGTGTTC
 N  C  N  V  A  H  K  N  I  R  E  Q  G  R  F  L  Q  D  V  F

ACCACGCTGGTGGACCTCAAGTGGCCACACACATTGCTCATCTTCACCATGTCCTTCCTG
 T  T  L  V  D  L  K  W  P  H  T  L  L  I  F  T  M  S  F  L

TGCAGCTGGCTGCTCTTCGCCATGGCCTGGTGGCTCATCGCCTTCGCCCACGGTGACCTG
 C  S  W  L  L  F  A  M [A] W  W  L  I  A  F  A  H  G  D  L

GCCCCCAGCGAGGGCACTGCTGAGCCCTGTGTCACCAGCATCCACTCCTTCTCGTCTGCC
 A  P [S] E  G  T [A][E] P  C  V  T  S  I  H  S  F  S  S  A

TTCCTTTTCTCCATTGAGGTCCAAGTGACTATTGGCTTTGGGGGGCGCATGGTGACTGAG
 F  L  F  S  I  E  V  Q  V  T  I  G  F  G  G  R  M  V  T  E

GAGTGCCCACTGGCCATCCTGATCCTCATCGTGCAGAACATCGTGGGGCTCATGATCAAC
 E  C  P  L  A  I  L  I  L  I  V  Q  N  I  V  G  L  M  I  N

GCCATCATGCTTGGCTGCATCTTCATGAAGACTGCCCAAGCCCACCGCAGGGCTGAGACC
 A  I  M  L  G  C  I  F  M  K  T  A  Q  A  H  R  R  A  E  T

CTCATCTTCAGCAAGCATGCGGTGATCGCTCTGCGCCACGGCCGCCTCTGCTTCATGCTA
 L  I  F  S  K  H  A  V  I [A] L  R  H  G  R  L  C  F  M  L

CGTGTGGGTGACCTCCGCAAGAGCATGATCATCAGCGCCACCATCCACATGCAGGTGGTA
 R  V  G  D  L  R  K  S  M  I  I  S  A  T  I  H  M  Q  V  V

CGCAAGACCACCAGCCCCGAGGGCGAGGTGGTGCCCCTCCACCAGGTGGACATCCCCATG
 R  K  T  T  S  P  E  G  E  V  V  P  L  H  Q  V  D  I  P  M
```

Fig 7 (cont)

```
GAGAACGGCGTGGGTGGCAACAGCATCTTCCTGGTGGCCCCGCTGATCATCTACCATGTC
 E  N  G  V  G  G  N [S] I  F  L  V  A  P  L  I  I  Y  H  V

ATTGATGCCAACAGCCCACTCTACGACCTGGCACCCAGCGACCTGCACCACCACCAGGAC
 I  D [A] N  S  P  L  Y  D  L  A  P  S  D  L  H  H  H  Q  D

CTCGAGATCATCGTCATCCTGGAAGGCGTGGTGGAAACCACGGGCATCACCACCCAGGCC
 L  E  I  I  V  I  L  E  G  V  V  E  T  T  G  I  T  T  Q  A

CGCACCTCCTACCTGGCCGATGAGATCCTGTGGGGCCAGCGCTTTGTGCCCATTGTAGCT
 R  T  S  Y  L  A  D  E  I  L  W  G  Q  R  F  V  P  I  V  A

GAGGAGGACGGACGTTACTCTGTGGACTACTCCAAGTTTGGCAACACCATCAAAGTGCCC
 E  E  D  G  R  Y  S  V  D  Y  S  K  F  G  N  T  I  K  V  P

ACACCACTCTGCACGGCCCGCCAGCTTGATGAGGACCACAGCCTACTGGAAGCTCTGACC
 T  P  L  C  T  A  R  Q  L  D  E  D [H] S  L  L [E] A  L  T

CTCGCCTCAGCCCGCGGGCCCCTGCGCAAGCGCAGCGTGCCCATGGCCAAGGCCAAGCCC
 L  A  S [A] R  G  P  L  R  K  R  S  V [P][M] A  K  A  K  P

AAGTTCAGCATCTCTCCAGATTCCCTGTCCTGAGCCATGGTCTCTCGGGCCCCCCACACG
 K  F  S  I  S  P  D  S  L  S  *

CGTGTGTACACACGGACCATGTGGTATGTAGCC
```

☐ *mark those amino acids which differ from the amino acids present at equivalent residues in the mouse sequence as shown in Fig. 1.*

Fig 8

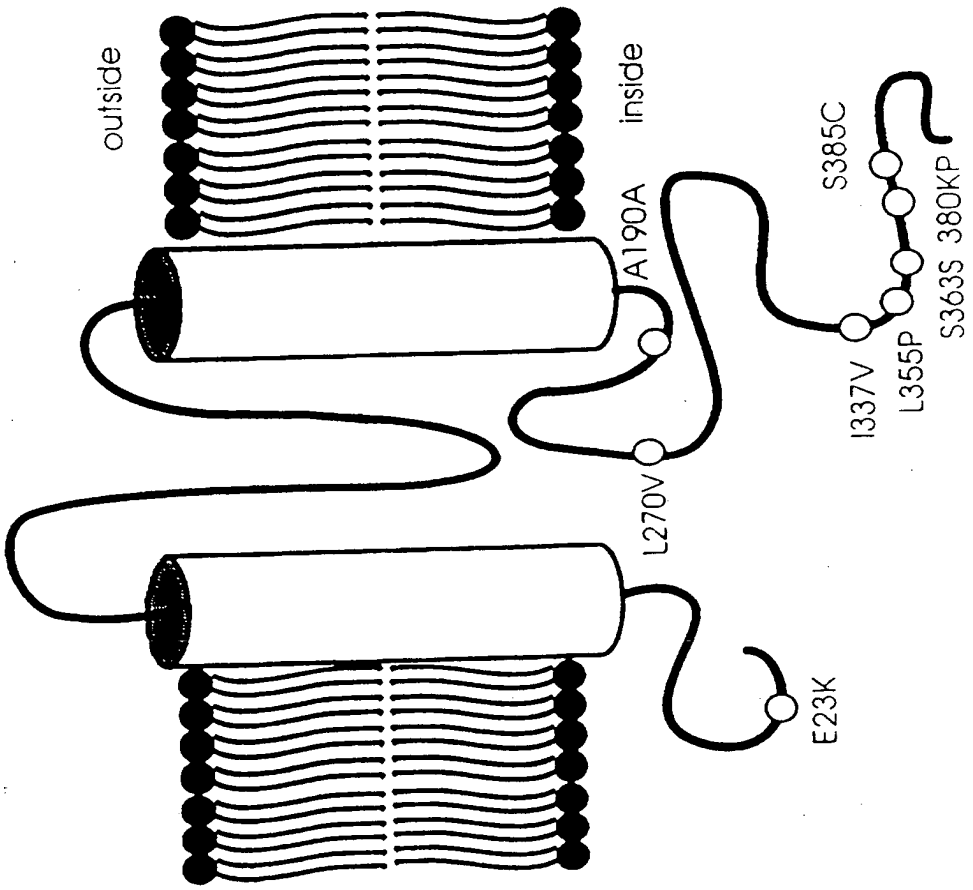
outside
inside
A190A
S385C
S380KP
S363S
L355P
I337V
L270V
E23K

Fig 9

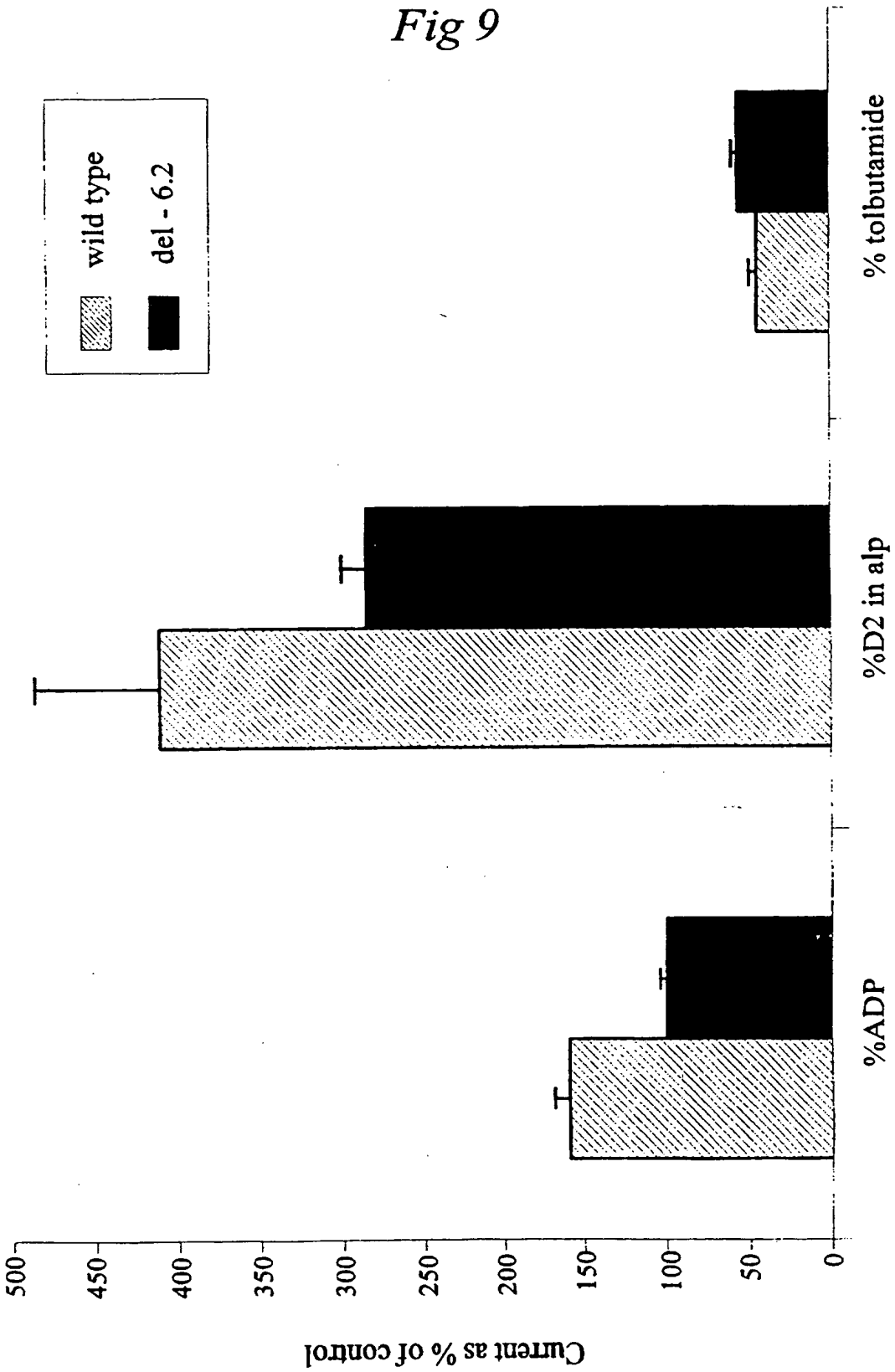
wild type
del - 6.2
% tolbutamide
%D2 in alp
%ADP
Current as % of control
500
450
400
350
300
250
200
150
100
50
0

Fig 10

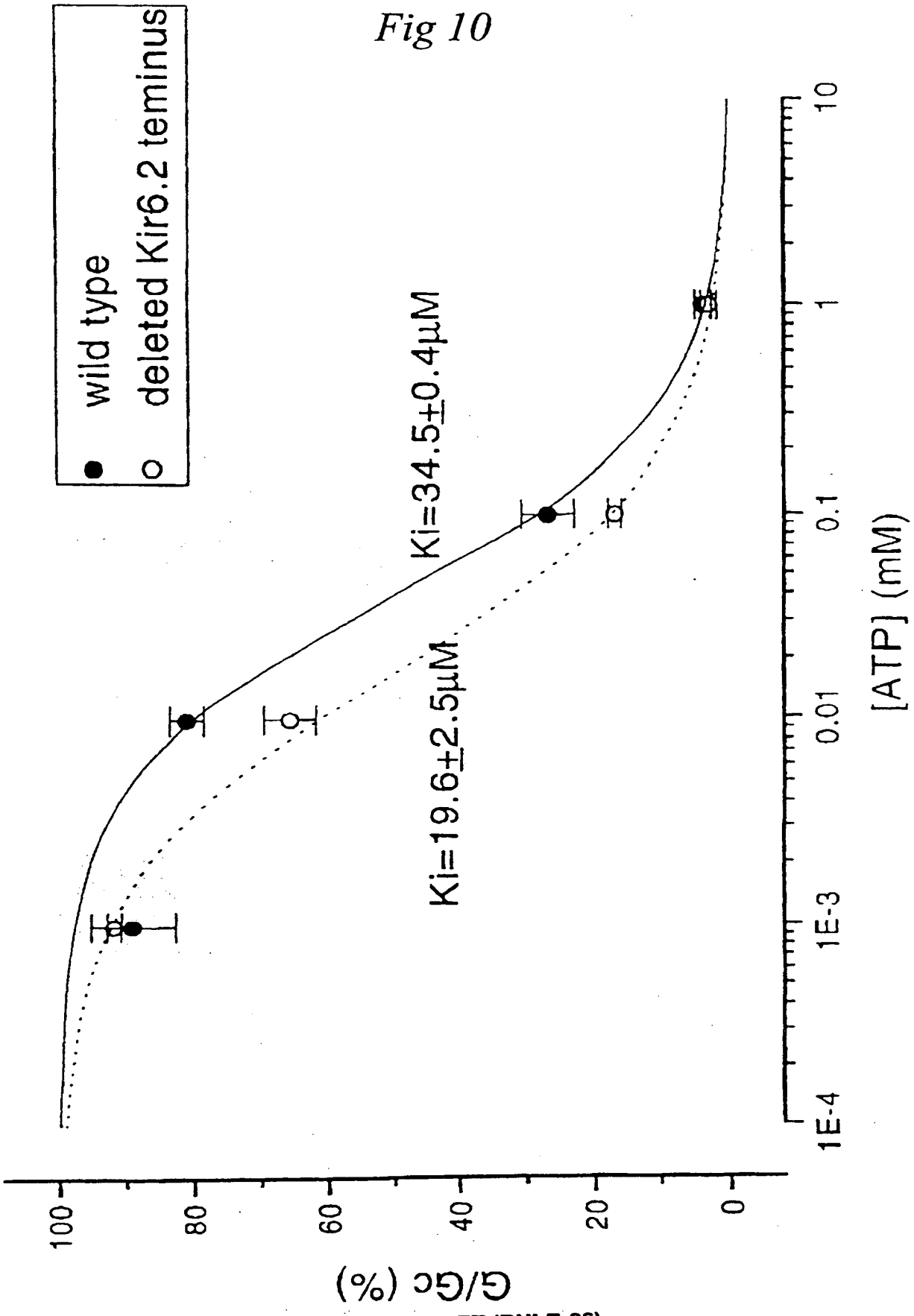
wild type
deleted Kir6.2 teminus
Ki=34.5±0.4μM
Ki=19.6±2.5μM
100
80
60
40
20
0
G/Gc (%)
1E-4
1E-3
0.01
0.1
1
10
[ATP] (mM)

Fig 11

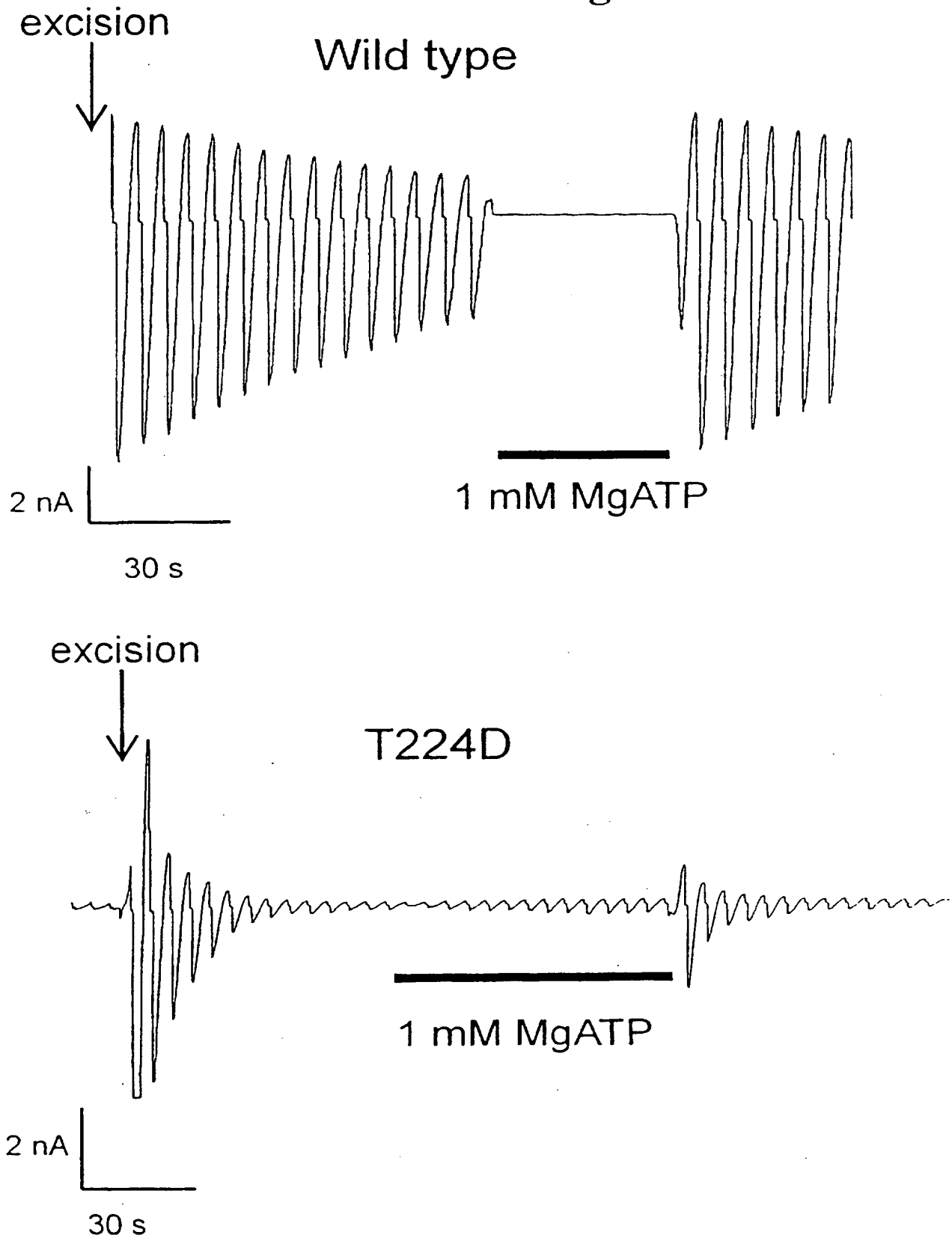
excision
Wild type
2 nA
30 s
1 mM MgATP
excision
T224D
1 mM MgATP
2 nA
30 s

Fig 12

Human Kir 6.2 promoter sequence

```
1   GGATCCGGAA AGCGGCGGGG GCGCTCCGGG AGGGGTGGAG TAGGACATAG
51  GGGGCGCACC TGGAGGAGAG ACGGGGCGGG GGTGGCCAGG ACCTGAGCTG
101 GAGCCTGGGA GCCCGAAGCC AGACAGGTGA GGCGGGAGAC CCGGAGGTGG
151 GGGTGAGGTC CGGTTAGTGG GAGAGATCCG GAGGTGTTAA GTTCTGAGCT
201 GGGCTGGGAA GGCAGGCAGG CTGGGCGGGG AGAAAGGCTC TTAGCGGGAG
251 GCCCAGGGGT GGTCAGCTGG TGGGGGAAGC TGGGGGAGGA CGCAGGGCCA
301 GGTGGAGAGC CGGCAGGGTT GGGGGCTCCC TAGGCGCCAG GCAGGTGGGC
351 TCAAGGGTGA GGCTGTTTTT TTTGTTTTGT TTTTTGTTTT TGAGACGGAG
401 TCTCGCTCTG TCGCCCAGGC TGGAGTGCAG TGGCGTGATC TTGGCTCACT
451 GCAACCTCCG CCTCTCGGGT TCAAGCGATT CTCCTGCCTC AGCTTCCTGA
501 GTAGCTGGGA TTACAGGTGC GCACCACCAT GCCCGGCTAA CTTTTGTATT
551 TTTAGTAGAG ATGGGGTTTC ACCATGTTGG TCAGGCTGGT CTCGAACTCC
601 TGACCTAGTG AGCTGGCCTC CTCAGCCTCC CAACGTACTG GGATTACAGG
651 CGTGAGCCAC CGCGCCCGGC
```

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(57) Abstract

The invention concerns materials and methods relating to an ATP-sensitive potassium ion channel (a K-ATP channel) molecule (and subunits thereof) which is implicated in a number of disease states associated with abnormal coupling of cellular metabolism to K^+ fluxes and/or electrical activity.

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INTERNATIONAL SEARCH REPORT

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PCT/GB 96/02831

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C12N5/10 C12Q1/68 A61K38/17

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X	WO 94 28131 A (CALIFORNIA INST OF TECHN) 8 December 1994 see claim 8	7
A	WO 95 28411 A (BAYLOR COLLEGE MEDICINE ;UNIV TEXAS (US)) 26 October 1995 see claims 1-50	17,19
E	EP 0 764 721 A (SUSUMU SEINO INOHANA SHUKUSHA ;JAPAN CHEM RES (JP)) 26 March 1997 see the whole document	1-23
P,X	SCIENCE (WASHINGTON, D. C.) (17-11-1995), 270(5239), 1166-70 CODEN: SCIEAS;ISSN: 0036-8075, XP002030881 INAGAKI, NOBUYA ET AL: "Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor" see the whole document	1-18
P,X	FEBS LETT. (1995), 377(3), 338-44 CODEN: FEBLAL;ISSN: 0014-5793, XP002030942 SAKURA, H. ET AL: "Cloning and functional expression of the cDNA encoding a novel ATP - sensitive potassium channel subunit expressed in pancreatic.beta.-cells, brain, heart and skeletal muscle" see the whole document	1-18

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9428131 A	08-12-94	EP 0714437 A	05-06-96
WO 9528411 A	26-10-95	AU 2285595 A	10-11-95
		CA 2187945 A	26-10-95
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		CA 2181880 A	19-03-97

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